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In re Patent Application of: Alitalo et al.

Application No.: 09/765,534 Confirmation No.: 1420

Filed: January 19, 2001 Art Unit: 1646

For: Flt4 (VEGFR-3) as a Target for Tumor Imaging and Anti-Tumor Therapy Examiner: Murphy, Joseph F.

DECLARATION OF KARI ALITALO, PURSUANT TO 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Kari Alitalo, do hereby declare and state as follows:

DECLARANT BACKGROUND

1. I am one of the inventors of the subject matter claimed in U.S. Patent Application No. 09/765,534 (referred to herein as "the patent application") [and all parent applications, *e.g.*, U.S.S.N. 07/959,951, filed October 9, 1992].

2. I am presently a Research Professor with The Finnish Medical Research Council of the Finnish Academy of Sciences. Since receiving my M.D. and M.Sc.D. in 1977 and 1980, respectively, from the University of Helsinki, I have worked substantially continuously as a professor and scientific researcher in Finland in areas of cellular and molecular biology and cancer research. My research has included substantial studies and explorations in fields of cancer, cancer metastasis, angiogenesis, and lymphangiogenesis. Through this research, I have gained extensive experience working with recombinant DNA/protein expression, analysis, and manipulation both *in vitro* and *in vivo*. My experience includes making and using antibodies raised against protein antigens.

3. In addition to my own research efforts and my collaborations with others, I receive numerous invitations to speak at national and international symposiums in

these areas of study, I supervise post-graduate research of others, I have authored and co-authored numerous original research articles published in peer-reviewed journals, and I have served on the editorial board of such journals. My *curriculum vitae* is attached hereto as Exhibit 1.

4. As shown in my *curriculum vitae*, I also have conducted and collaborated in substantial research relating to the Flt4 receptor tyrosine kinase, also known as "Vascular Endothelial Growth Factor Receptor-3" or "VEGFR-3." This receptor is a member of a subfamily of class III tyrosine kinase receptors that also includes VEGFR-1 (also known as Flt1) and VEGFR-2 (also known as KDR). The human Flt4 receptor has at least two sequences a "short" form (SEQ ID NO: 2) and "long" form (SEQ ID NO: 4), differing in their C-terminal region, but having the same extracellular domain. "Vascular Endothelial Growth Factor C" or "VEGF-C" acts as a high affinity ligand for the VEGFR-3 receptor, binding and stimulating phosphorylation of VEGFR-3. I have co-authored publications and filed patent applications relating to Flt4/VEGFR-3. Thus, my laboratory, my collaborators, and I have substantial expertise and experience working with and expressing the Flt4 receptor, fragments of that receptor, and antibodies specific for the same.

DISCUSSION OF CLAIMED SUBJECT MATTER

5. I understand that we are claiming in the patent application an isolated peptide or protein comprising a member selected from the group consisting of:

(a) the Flt4 receptor tyrosine kinase (Flt4) extracellular domain amino acid sequence set forth in SEQ ID NO: 2 or 4 and;

(b) a fragment of said extracellular domain, wherein the fragment includes sufficient amino acid sequence of SEQ ID NO: 2 or 4 to generate an immune response in a nonhuman mammal to produce antibodies that **specifically** bind to Flt4 (SEQ ID NO: 2 or 4). Before 1992 and today, scientists in my field understood the term "specifically binds" to refer to antibodies that immunoreact with one antigen (*e.g.*, Flt4) and fail to appreciably cross-react with other known, related antigens (such as the Flt1 and KDR sequences that were known at the time we filed the first priority application in 1992). Anti-Flt4 antibodies and uses thereof are described in the patent application. *See, e.g.*, the specification at top of page 34 and at page 35.

7. I understand that we are claiming peptides that comprise fragments of the Flt4 amino acid sequence (SEQ ID NO: 4) defined by cyanogen bromide (CNBr) cleavage sites that exist in the Flt4 sequence; specifically, we are claiming fragments that contain some sequence from the extracellular domain of Flt4. The patent application describes CNBr cleavage of Flt4 at page 32, lines 9-14. CNBr cleaves polypeptides at the C-terminal end of methionine residues. *See, e.g.*, Exhibit C, Stryer page 55; Exhibit B, Sambrook, page 17.29. The Flt4 peptides defined by CNBr cleavage sites and that contain extracellular domain amino acids are shown in Exhibit D.

INFORMATION RELEVANT TO THE ENABLEMENT REJECTION

8. I understand that the Patent Office has rejected certain claims in the present application, alleging that the patent application does not adequately teach a practitioner with average experience in the field of the invention to make and use the invention in a manner commensurate in scope with the claims. The Patent Office alleged lack of enablement for a Flt4 fragment encoded by 200 nucleotides of SEQ ID NO: 1 or 3, a polypeptide comprising a Flt4 extracellular domain fragment, and a polypeptide comprising Flt4 peptides obtained by CNBr cleavage. I am providing this declaration to provide information relevant to overcoming the rejection.

9. The present application teaches how to make and use not only Flt4 polypeptides, but also fragments and variants thereof. *See, e.g.*, specification at page 32, lines 1-14. By 1992, scientists were able to produce peptides by both chemical synthesis and through *in vitro* and cellular expression systems. *See, e.g.*, Exhibit C, Stryer, pages 66-67, and Exhibit B, Sambrook, chapters 16 and 17. Peptides could also be produced from larger polypeptides/proteins using digestion with various agents, *e.g.*, cyanogen bromide (CNBr) as discussed above.

10. The specification describes Flt4 peptides and their use in producing both polyclonal and monoclonal antibodies, *see, e.g.*, page 25, lines 25-27, and page 33, lines 3-23. Techniques for producing both monoclonal and polyclonal antibodies were well known by 1992. *See generally* Exhibit A, Harlow, and Exhibit B, Sambrook, pages 18.3-18.18. Likewise, techniques to select an antibody that was immunospecific for one protein, and which failed to cross-react with other proteins, were well known. Western blotting provides one way of testing the specificity of a peptide and antibodies that specifically bind to that

peptide. *See, e.g.*, Exhibit C, Stryer, page 63; Exhibit B, Sambrook, pages 18.60-18.61. Other available immunoassays include radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). *See, e.g.*, Exhibit B, Sambrook, pages 18.19-18.25 and Exhibit C, Stryer, page 63. Such assays lend themselves to automation, allowing rapid screening of antibodies. It was well known that cross-reactive antibodies could more easily be avoided by immunizing with peptides that had sequence unique to the desired antigens, rather than peptides whose complete sequence was shared by two or more proteins. To identify peptides with unique sequence, a practitioner could have used a sequence alignment between the Flt4 sequence taught in the specification and other known protein sequences. The patent application provides an alignment between VEGFR-1 and Flt4. *See* Figs. 2A-F and at page 24, lines 12-13. One could also make a similar alignment between Flt4 and VEGFR-2 (or what sequence of VEGFR-2 was known in 1992), or a three way alignment between the three family members. Computer software existed in 1992 to permit scientists to generate such alignments rapidly.

INFORMATION RELEVANT TO THE PRIOR ART REJECTION

11. I also understand that the Patent Office has alleged that some of the claimed subject matter is not novel in view of Terman et al., Oncogene, 6: 1677-1683 (1991). I am further providing this declaration to provide information relevant to overcoming that rejection. Some of the claims specify a Flt4 extracellular domain fragment with sufficient Flt4 sequence to generate an immune response that is specific to Flt4. These requirements exclude peptides that consist of perfect sequence identity with the Terman sequence or hypothetical fragments of the Terman KDR sequence. If one immunized a mammal with KDR peptides, one would expect to generate antibodies that would bind KDR specifically. One might also generate antibodies that bound KDR as well as other proteins, *i.e.*, nonspecifically. However, a practitioner could not have produced and cannot produce an antibody that specifically binds Flt4 using only the KDR sequence taught by Terman or any later known KDR sequence, *e.g.*, that used by the Office in its "Sequence Comparison A."

12. Terman also fails to disclose a peptide sequence that matches a Flt4 fragment defined by CNBr cleavage sites and that contains Flt4 extracellular domain sequence. Exhibit D shows Flt4 fragment amino acid sequences with stop/start sites corresponding to positions of cyanogen bromide cleavage of Flt4 receptor tyrosine kinase and

comprising at least one extracellular domain amino acid. Exhibit E provides a superposition of the fragments of Exhibit D on the Sequence Comparison A provided with the Office action. This comparison shows that the complete sequence of each of these Flt4 fragments is divergent from (non-identical to) the KDR sequence.

BIBLIOGRAPHY

13. In the above discussion, I have made reference to several publications that were widely known and available to scientists in my field by 1992:

Exhibit A) Harlow, E. et al., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, 1988 [hereinafter, Harlow].

Exhibit B) Sambrook, J., et al., "Molecular Cloning A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, 1989 [hereinafter, Sambrook].

Exhibit C) Stryer, L., Biochemistry, 3rd ed., Freeman, 1988 [hereinafter, Stryer].

Selected, relevant pages from these publications have been provided in the attached Exhibits.

OATH/VERIFICATION

14. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief and believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 26.10.04 Signature:

Antibodies

A LABORATORY MANUAL

Ed Harlow
Cold Spring Harbor Laboratory

David Lane
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Cold Spring Harbor Laboratory
1988

EXHIBIT A

Antibodies
A LABORATORY MANUAL

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CONTENTS

The major problem that is encountered when preparing anti-peptide antibodies is whether they will recognize the native protein. Assays that need or benefit from anti-native antibodies, such as immunoprecipitation, many cell staining techniques, or immunoaffinity purification, will succeed only when the peptide sequence is displayed on the surface of the native molecule in a conformation similar to the peptide–carrier conjugate. Therefore, the successful production of anti-peptide antibodies is often determined by the researcher's ability to predict the location of certain peptide sequences in the three-dimensional structure of the protein.

Because of their size, peptides may not be immunogenic on their own. To elicit an antibody response directly, they must contain all of the features of any immunogen, notably they must have an epitope for B-cell binding and a site for class II–T-cell receptor binding. Some peptides, even surprisingly small ones, contain both these sites (or more properly, one sequence that can serve both functions), and these peptides can be used without carriers (e.g., see Beachy et al. 1981; Lerner et al. 1981; Dreesman 1982; Jackson 1982; Atassi and Webster 1983; Young et al. 1983). Unfortunately, there are no methods, short of immunization, to test this, and therefore, most peptides are coupled to carrier proteins before injection. An exciting recent development is the use of synthetic class II–T-cell receptor sites synthesized directly with the desired epitope (Francis et al. 1987; see also, Good et al. 1987; Borras-Cuesta et al. 1987; Leclerc et al. 1987). Although there are not enough cases to determine how widely applicable this approach will be, the concept is provocative. With this strategy, the peptide of interest is synthesized as either an amino- or carboxy-terminal extension of a known class II–T-cell receptor site. The synthetic peptide, now containing both sites, is injected without coupling and used to induce an antibody response. The first experiments using this approach look very promising, and this may become an important alternative to coupling with carrier proteins.

Peptides usually are synthesized with an automated machine using solid-phase techniques. The methods for synthesis and purification of the peptide are beyond the scope of this book. However, to judge the success of the coupling reaction and to determine the number of moles of peptide bound to the carrier, a small proportion of the peptide needs to be labeled. This can be done by including a small amount of ^{14}C-labeled amino acid in the synthesis or by iodinating a sample of the peptides on a tyrosine or histidine residue (see p. 324) after the synthesis. A small sample of these iodinated peptides can then be added to the coupling reactions to ascertain the success of the coupling.

During immunization, antibodies to the carrier proteins or the coupling agent will also be produced, and these are normally removed by affinity-purifying the anti-peptide antibodies on a column prepared with conjugates of the peptide and a second carrier molecule. Techniques for affinity purification of the antibodies are described in general on p. 313.

Designing the Peptide

Probably the most frequently asked question concerning synthetic peptides is what sequence should be used for the immunogen (reviewed in Doolittle 1986). Although there is no one correct answer, enough anti-peptide antibodies have been raised to make suggestions for peptide choices. However, preparing anti-peptide antibodies is still an empirical exercise. What works well for one immunogen may fail completely for another.

Choosing the Appropriate Peptide Sequence

With careful synthesis, coupling, and immunizations, most sequences can be used to induce antibodies specific for the peptide itself. When considering which sequence to use, most people actually want to know how likely will it be that the anti-peptide antibodies will recognize the native protein. Early work suggested that peptides containing hydrophilic amino acids (Hopp and Woods 1981, 1983; Kyte and Doolittle 1982) and proline residues were more likely to be exposed on the surface of the native protein than other sequences, and many peptides have been prepared using these criteria. In assessing the value of these criteria, hydrophilicity is required but is not sufficient to predict the surface location of a particular sequence. Many strongly hydrophilic amino acid sequences are buried in water pockets or form inter- or intramolecular bonds and are thus excluded from interactions with anti-native antibodies. Therefore, hydrophilicity can be thought of as required but not sufficient for choosing peptide sequences (see p. 661 for hydrophilicity values). Hydrophilic peptides are also more likely to be soluble for coupling reactions.

The presence of proline residues in synthetic peptides originally was suggested because β-turns often form portions of known epitopes. However, the presence of proline residues in peptides does not have much predictive value when antisera are tested for binding to the surface of native proteins. Although many excellent anti-peptide antisera have been prepared against sequences with proline residues, there is not sufficient evidence to target prolines when designing peptides.

More recently, several workers have noted that carboxy-terminal sequences often are exposed and can be targeted for anti-peptide sequences. Although using carboxy-terminal sequences does not guarantee that the resulting antibodies will recognize the native protein, a surprisingly high percentage will. Similarly, many amino-terminal regions are exposed, and these also may make good targets.

Another potentially useful parameter for selecting peptide sequences is the "mobility" of the amino acid residues. Originally, it was noted that the regions of a protein that become epitopes often have a higher temperature than other regions, as determined by NMR and X-ray structure (Moore and Williams 1980; Robinson et al. 1983; Tainer et al. 1984; Westhof et al. 1984). Higher temperature in crystallography and NMR distinguishes regions that are more mobile from

regions that are more static. These observations have led to the suggestion that stretches of amino acids that are more flexible are more likely to be epitopes. In the preparation of anti-peptide antibodies, when a peptide is coupled to a carrier molecule, it has a different local environment than in the original protein. When choosing a sequence for antibody production, a region of the protein that is more flexible will be more likely over time to form a structure that is similar to the peptide–carrier conjugate. Although the measure of mobility may become a useful criterion for selecting good peptide sequences, it has not been tested in enough detail to determine whether it will have any predictive value.

At present, a reasonable order of suggestions for choosing peptide sequences would be:

1. If possible, use more than one peptide.
2. Use the carboxyl-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
3. Use the amino-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
4. Use internal hydrophilic regions; perhaps using longer peptides.

Size of the Peptide

The smallest synthetic peptides that will consistently elicit antibodies that bind to the original protein are 6 residues in length. Responses to smaller peptides are typically weak or will not recognize the protein of interest, either in a native or denatured state. Since epitopes consisting of smaller regions have been reported, the lower limit presumably reflects the difficulty of recognizing the smaller peptides coupled to carriers. With peptides of 6 amino acids or slightly larger, the responses vary. Some will generate good antibodies and some will not. Generally, peptides of approximately 10 residues should be used as a lower limit for coupling.

In the literature two strategies are suggested for peptide length. One school suggests using long peptides (up to 40 amino acids long) to increase the number of possible epitopes, while other authors argue that smaller peptides are adequate and their use ensures that the site-specific character of anti-peptide antibodies is retained. Both strategies have been used successfully. Two important preliminary questions to consider are: (1) Does the anti-peptide serum need to recognize the native protein? If so, use longer peptides or prepare anti-peptide antisera against multiple peptides. (2) How good is your peptide synthesis facility? Peptides over 20 residues in length are increasingly difficult to synthesize, yielding products with inappropriate side reactions. Longer peptides also are more likely to contain residues that make the coupling to carrier molecules more difficult. The correct decison between peptides with 10–15 residues and longer peptides will depend on the experimental design and will normally be a compromise between these factors. The safest choice, but also the most expensive, will be to prepare multiple small peptides of 10–15 amino acids in length from various regions of the sequence.

Coupling Strategy

When choosing the sequence for a synthetic peptide, one factor that often is overlooked is the method of coupling. Most coupling methods rely on the presence of free amino, sulfhydryl, phenolic, or carboxylic acid groups. Free amino groups used for coupling will be found on lysine side chains or on the amino-terminal residue. Sulfhydryl groups are found on cysteine side chains, phenolic groups on tyrosines, and carboxylic acid groups on aspartic acids, glutamic acids, and the carboxy-terminal residue. Coupling methods should be used that link the peptide to the carrier via either the carboxy- or amino-terminal residue. When preparing antibodies against the carboxy-terminal region of the protein, the coupling should be done through the amino terminus of the peptide. Similarly, the coupling for amino terminal fragments should be done through the carboxy-terminal region of the peptide. For internal fragments, the major consideration is that the peptide be coupled by an end and not through a central residue.

The easiest strategy to manipulate the type of coupling is to add an extra amino acid on either the amino or carboxyl terminus to allow simple, one-site coupling to the carrier. Any coupling method that potentially can bind to an internal residue should be avoided. Similarly, coupling methods should be chosen that will bind to only one amino acid, if possible. If multiple coupling sites are possible, they should be localized to either the amino or carboxyl terminus, and the coupling should be adjusted to link only through one site per peptide on average. It is important to remember that it is often easier to use different peptides than design elaborate coupling schemes.

Choosing the Appropriate Carrier

Many different carrier proteins can be used for coupling with synthetic peptides. The two most commonly used are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). Both work well in most cases, but each has disadvantages. Because of its large size, KLH is more likely to precipitate during cross-linking, and this can make handling KLH difficult in some cases. On the other hand, BSA is very soluble, but often is a good immunogen in its own right. For most purposes, either carrier will be adequate. Use whichever is more convenient.

Three other carriers that are used occasionally are ovalbumin, mouse serum albumin, or rabbit serum albumin. Ovalbumin can be used as a good carrier for most purposes. It is also a good choice for a second carrier when checking that antibodies are specific for the peptide itself and not the carrier. MSA or RSA may be used when the antibody response to the carrier molecule must be kept to a minimum.

BSA has 59 lysine (30–35 are available for coupling), 19 tyrosine, 35 cysteine, 39 aspartic acid, and 59 glutamic acid residues. Ovalbumin has 20 lysine, 10 tyrosine, 6 cysteine, 14 aspartic acid, and 33 glutamic acid residues.

3

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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EXHIBIT B

Molecular Cloning
A LABORATORY MANUAL
SECOND EDITION

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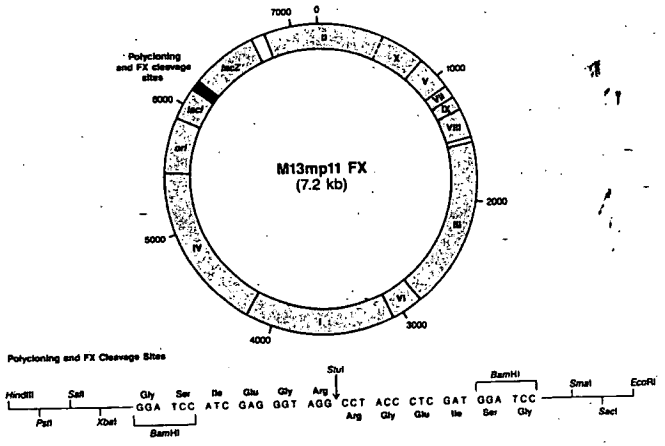

FIGURE 17.10
M13mp11 FX is an M13mp11 derivative that has the polycloning site oriented with *Eco*RI adjacent to *lacZ'*. DNA encoding the recognition sequence for blood coagulation factor X_a (Ile Glu Gly Arg [shown in bold]) is cloned into the *Bam*HI site. Any coding sequence with blunt ends can be cloned into the *Stu*I site to place the FX_a cleavage site adjacent to the desired protein sequence. The entire DNA sequence can then be inserted into an appropriate expression vector (e.g., pLcII) (Nagai and Thøgersen 1984).

ALTERNATIVE EXPRESSION SYSTEMS

In some cases, the methods described above do not result in the production of the desired protein. In particular, it may be important that the protein not have an amino-terminal methionine, which is coded for by the ATG in these vectors. The amino-terminal methionine is removed in *E. coli* to different extents for different proteins. Another common problem is that some proteins produced intracellularly are inactive, perhaps due to incorrect folding. Some foreign proteins are degraded by intracellular proteases. Two other expression systems may solve some of these problems.

Expression of a Cloned Gene as Part of a Fusion Protein That Can Be Cleaved by a Protease or Cyanogen Bromide

The cloned gene can be expressed as part of a fusion protein composed of a prokaryotic amino terminus connected to the protein of interest by a sequence that can be cleaved by a protease or by cyanogen bromide (Itakura et al. 1977; Goeddel et al. 1979; Nagai and Thøgersen 1984; Sung et al. 1986). The fusion protein is often stable compared to the foreign protein, and a protein can be made that does not contain an amino-terminal methionine.

Nagai and Thøgersen (1984) have developed a vector designed to produce hybrid proteins that can be cleaved with the blood coagulation factor X_a to liberate the foreign protein. Factor X_a cleaves specifically after the arginine of the tetrapeptide Ile-Glu-Gly-Arg, which connects the 31 amino-terminal residues of the bacteriophage λ cII protein to the protein of interest (Figure 17.10).

Other proteases have been similarly employed. For example, Jia et al. (1987) used λgt11 to make a β-galactosidase–carbohydrate-binding protein 35 (CBP35) fusion protein, which was then cleaved with V8 protease to release CBP35.

Proinsulin is readily degraded in *E. coli*. However, expression of a fusion protein composed of two molecules of proinsulin has been shown to stabilize the protein, perhaps because the protein product is larger than proinsulin. This fusion protein can then be cleaved by cyanogen bromide (Shen 1984).

In each case, it is important that the target amino acids for the protease or cyanogen bromide (which cleaves after methionine residues) does not occur at other locations within the protein of interest.

The scope of recombinant DNA research has undergone a dramatic expansion. In its early days, the field was concerned mainly with describing the structure of eukaryotic cDNAs, establishing the topography of the corresponding genomic sequences, and defining the location of controlling elements. Although this kind of cataloging remains an essential part of molecular cloning, the field has now broadened to include expression of cloned genes and analysis of the proteins they encode. Methods commonly used to express cloned genes in prokaryotic and eukaryotic systems are discussed elsewhere in this manual. In this chapter, we describe techniques to detect and quantitate foreign proteins synthesized in these systems. Although we emphasize methods to analyze proteins expressed in mammalian cells, the same techniques are applicable to proteins synthesized in yeast and prokaryotic systems. We also describe methods to synthesize proteins in vitro using the rabbit reticulocyte system and natural mRNAs or synthetic RNAs.

Proteins synthesized in heterologous systems can be detected either by assaying for a particular biological activity or by employing assays that are independent of such activity. In a few cases, the protein of interest carries an enzymatic or other biological activity (e.g., the ability to bind a specific ligand) that can be assayed in intact cells or in extracts in vitro. Although such assays can be extremely useful, they frequently suffer from one of several practical limitations: (1) They may not be sufficiently sensitive to detect the small amounts of protein that are synthesized in small-scale mammalian cultures. (2) The host cells may themselves express an endogenous protein that either displays the same biological activity as the foreign protein or interferes with it. (3) The detection of biological activity in an extract says nothing about the specific activity of the protein of interest (i.e., the activity displayed by a given amount of the protein). This point becomes especially important when measuring the activity of mutated forms of the protein that have been generated by in vitro mutagenesis or when expressing the wild-type protein in heterologous cells that may not allow the protein to fold correctly or may not carry out the correct posttranslational modifications. For these and other reasons, it is essential to develop assays that are independent of biological activity and sensitive enough to measure very small amounts of the protein. The reagents of choice for these assays are antibodies that react specifically with the foreign protein.

Production of Antibodies

It is important to bear in mind from the outset that antibodies that react specifically with the foreign protein fall into three general classes:

- *Antibodies that react with the foreign protein independently of its conformation.* Antibodies of this type are particularly useful for measuring the total amount of the target protein present in crude preparations or cell extracts. They are usually raised by immunizing animals with partially denatured protein or with a peptide whose sequence corresponds to part of the intact protein. However, monoclonal antibodies that are pan-specific are not uncommon.
- *Antibodies that react only with epitopes specific to the native form of the target protein.* Antibodies of this type are typically monoclonal and recognize a given sequence of amino acids only when it occurs in the particular three-dimensional conformation that is characteristic of the native form of the target protein.
- *Antibodies that react only with denatured forms of the protein.* These are raised against fully denatured antigens and can be either monoclonal or polyclonal.

Factors Affecting Immune Response

Although there is no way to guarantee the production of particular types of antibodies, it is advisable to choose an immunization regimen that will favor antibodies with the desired characteristics. It is then necessary to screen several independent antisera or a series of monoclonal antibodies to identify those best suited to the tasks at hand. A number of factors that affect the strength and specificity of the immune response should be considered by workers planning to raise antisera. These are described below.

SPECIES OF ANIMAL

To raise antisera that react with as many potential epitopes as possible, choose a species of animal that is phylogenetically different from the species from which the target protein was isolated. Inoculation of a native protein into an animal of the same species is likely to yield antibodies directed only against minor epitopes that vary from individual to individual. For most proteins, rabbits are the animals of choice: They are easy to bleed, yielding several milliliters of serum; they are sufficiently robust to withstand multiple bleedings; and they are reasonably inexpensive. Chickens have many of the same virtues; however, they are clearly a second choice because their antibodies, unlike those of rabbits, do not bind to protein A of *Staphylococcus aureus*. Mice and guinea pigs are less desirable because their small size makes repeated drawing of blood difficult.

GENETIC FACTORS

Outbred animals are preferred to inbred strains, which may be genetically incapable of responding vigorously to certain antigens. Even outbred animals, however, exhibit a broad range of intensity of response to the same immunogen. It is therefore essential to immunize several animals, especially when attempting to raise antisera against poor immunogens. In some cases, it is possible to improve the response to such antigens by physically coupling them to a carrier protein such as keyhole limpet hemocyanin (KLH) or bovine serum albumin.

PHYSICAL STATE OF THE ANTIGEN

Aggregated protein is a much better immunogen than soluble, dispersed protein. Partial denaturation may help to increase immunogenicity but may yield antisera that fail to react with native protein. Severely denatured proteins (e.g., proteins that have been extensively boiled, reduced, and alkylated) often yield antisera that react exclusively or preferentially with denatured protein. Many immunology textbooks state that denatured proteins are poor antigens. This is a fallacy that almost certainly stems from the failure to assay the antisera against the appropriately denatured form of the antigen.

When the amount of antigen is limiting, adjuvants are used to enhance the immune response. These substances prolong the half-life of the antigen by protecting it from degradation. In addition, they minimize any direct toxic effects of the antigen and, by allowing a slow, sustained release from the site of immunization, increase the efficiency of uptake of the antigen by macrophages. A large variety of substances can be used as adjuvants, including alum, mineral oils, charcoal, and Freund's adjuvant (complete or incomplete). Nitrocellulose filters and polyacrylamide are particularly useful adjuvants when only small amounts of antigen are available. Bands of polyacrylamide containing specific polypeptides can be excised from polyacrylamide gels or electrophoretically transferred to nitrocellulose filters and used directly for immunization (see discussion below on immunizing with small amounts of antigen).

Note: If the antisera will be used to detect proteins expressed in *E. coli*, Freund's adjuvant should not be used due to the presence of bacterial components in the Freund's adjuvant that will generate cross-reactive antibodies to *E. coli* proteins.

AMOUNT OF ANTIGEN

Injection of large amounts of a soluble immunogen can lead to the establishment of a tolerant state. Although the use of adjuvants greatly reduces the chances that tolerance will be induced, it is important not to use more antigen than necessary, since this often leads to the generation of antisera of low avidity. Although there is no foolproof method to predict what the lowest effective dose of an unknown antigen will be, the immunization regimens described below usually yield antisera of high titer and avidity. If sufficient animals and antigen are available, it is best to inoculate batches of animals with different amounts of antigen. Those that receive too little antigen to elicit a response can always be given a larger dose subsequently. Booster injections are essential to obtain antisera of high titer and avidity. The first of these booster injections should be given when the primary response is past its peak (about 4–6 weeks after the primary injection of antigen in the case of rabbits). About 7–10 days after the first booster injection, test bleeds should be taken, and further booster injections should be given at regular intervals to those animals that show the maximum response to the antigen. The length of these intervals varies from antigen to antigen and cannot be predicted in advance. The best procedure is to boost the animal when the titer of circulating antibody begins to drop. The amount of antigen used for boosting is usually two- to threefold less than that used for primary immunization. Such booster injections are usually given to rabbits by the same route (although not necessarily at the same site) as the primary immunization. However, mice are more frequently boosted by intravenous injection of soluble antigen even if the primary immunization was by another route.

ROUTE OF INJECTION

Antigens stimulate the strongest immune responses when they are injected directly into the animal's popliteal lymph nodes. However, this requires special skill, and the much simpler methods of injection of antigen mixed with adjuvant into multiple intradermal or subcutaneous sites or into a single intramuscular site are used more commonly. In multiple intradermal injections, where the antigen-adjuvant complex is injected at 20–40 sites spread over the body of the animal, the primary response is produced faster and the titer of antibody is generally higher than when the antigen is injected into a single intramuscular site. Furthermore, fewer booster injections are required. However, since an ulcer forms at the site of each injection of adjuvant, the multiple-injection method may cause more distress to the animal.

IMMUNIZATION SCHEDULES

The injection of antigen should be carried out by someone experienced in handling animals according to the procedures approved by your animal care facility. Some facilities prefer not to use certain types of adjuvants (e.g., Freund's complete adjuvant or crushed polyacrylamide gels), and it is therefore important to agree on an immunization regimen before animals are ordered. *Make sure that a sample of blood is drawn from each animal before immunization is begun.* Although the immunization schedule will vary according to the nature of the antigen, the amount of antigen available, and its immunogenicity, a reasonable general plan for raising antisera against an antigen that is readily available is described below.

Rabbits

Dissolve 100 μg of antigen in 0.5 ml of a buffer in which the antigen is soluble, and emulsify the solution with an equal volume of Freund's complete adjuvant. Use the emulsion immediately. An emulsion of the desired consistency is readily obtained by passing the solution through a double-canula microemulsifying needle (Thomas Scientific 345OD46).

- *For intramuscular injection:* Inject 0.5 ml of emulsified antigen-adjuvant into each of two limbs of the animal. Four to 6 weeks later, inject 0.25 ml of freshly prepared emulsion into another limb. Limbs should be used in rotation every 4–6 weeks for subsequent booster injections.
- *For intradermal injection:* Inject 0.025 ml of emulsified antigen at 24–36 sites distributed over the back of a shaved rabbit. Four to 6 weeks later, boost the animal by intramuscular injection of 0.25 ml of freshly prepared emulsified antigen-adjuvant into each of two limbs of the animal.

Arrange for blood (20–40 ml) to be drawn 7–10 days after each booster injection. The serum should be tested for the presence of antibody by one of the standard methods, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), or immunoprecipitation. Animals that respond poorly to the antigen after two booster immunizations should be sacrificed. Responsive animals should be boosted at regular intervals (2–3 months) until a high titer of antibody is attained. Blood (40 ml) can then be drawn weekly until the titer drops. The animal should then be allowed to rest for 2–3 months and reboosted before bleeding is resumed.

Mice

Dissolve 20–50 μg of antigen in 100 μl of a buffer in which the antigen is soluble, and emulsify the solution with an equal volume of Freund's complete adjuvant. Use the emulsion immediately. Inject 50 μl of the emulsified antigen-adjuvant subcutaneously at each of four sites. Four to 6 weeks later, boost the mice with an intraperitoneal injection of 100 μl of the appropriate buffer containing 5–10 μg of soluble antigen. The mice should be bled from the median tail vein 7–10 days after the booster injection, and the serum should be tested for the presence of antibody by one of the standard methods. The animals can be reboosted if necessary, and blood can be withdrawn every 3–4 days until the antibody titer drops.

Immunizing with Small Amounts of Antigen

Frequently, the molecular biologist is faced with the problem of raising antibodies to proteins that are available in extremely small amounts. To maximize the chances of obtaining an immune response, the soluble antigen (up to 10 μg) emulsified in Freund's complete adjuvant should be injected into the popliteal lymph node of a rabbit. However, immune responses can also be obtained when animals are immunized by conventional routes with small amounts of polypeptides (100 ng to 5 μg) that have been recovered from polyacrylamide gels. After electrophoresis, the polypeptides are transferred from the gel to a nitrocellulose filter by western blotting, and the relevant segment of the nitrocellulose filter is then either implanted subcutaneously in a rabbit or dissolved in a small volume of dimethyl sulfoxide (DMSO), mixed with Freund's complete adjuvant, and injected intradermally as described above. Alternatively, the band of polyacrylamide containing the polypeptide of interest is cut from the gel. The polyacrylamide is then broken into small fragments by freezing and thawing and passage through a narrow-gauge hypodermic needle. The fragments of gel are then emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously into mice or intradermally at many sites into a rabbit.

Monoclonal Antibodies

The introduction of cell hybridization to generate myeloma-like cell lines that secrete monoclonal antibodies with desired specificities has greatly expanded the range, number, and reproducibility of immunological reagents (Köhler and Milstein 1975, 1976). Although the protocols to develop hybridomas are nearly as numerous as the number of investigators using them, almost all of them involve fusing spleen cells from a hyperimmunized donor with cells from a genetically marked myeloma that has been adapted to grow in tissue culture. Mixed populations of the resulting hybrid cells are tested for their ability to secrete antibody of the desired specificity, and clones derived from individual cells are then established. In the best cases, the supernatant of the resulting cultures contains up to 50 μg/ml of immunoglobulin. However, even greater amounts of antibody (5–20 mg/ml) can be obtained by growing the cloned hybridoma as an ascites tumor in the peritoneal cavity of mice.

Monoclonal antibodies are particularly useful when an immune reagent is required that reacts specifically with a single component in a complex mixture of antigens, for example, an enzyme that is contaminated with other proteins. Although an immunized animal may develop antibodies against all of the proteins in the mixture, an individual B lymphocyte will produce antibody specific for only one of them. The establishment of permanent cell lines from such individual B cells provides a method to obtain an antibody that is specific for the enzyme of interest. When using a monoclonal antibody, it is important to determine its subclass, using commercially available anti-idiotypic reagents, since not all subclasses of immunoglobulins bind efficiently to commonly used immunoabsorbents such as staphylococcal protein A.

A description of methods to generate monoclonal antibodies is beyond the scope of this manual. However, a number of excellent reviews are available that describe the techniques in detail (see, e.g., Campbell 1984; Goding 1986; Kipps and Herzenberg 1986; Harlow and Lane 1988).

Raising Antisera against Synthetic Peptides

If the amino acid sequence of the protein of interest is known or can be deduced from the corresponding nucleic acid sequence, it is possible to raise specific antisera by immunizing animals with short synthetic peptides (for review, see Lerner 1984). If the experimenter wishes to generate antibodies that react with specific sites in the target protein, there may be little or no choice in the sequence of the peptide that is used as an immunogen. However, if the antibody is to be used merely as a reagent to identify the protein, there may be an opportunity to select a sequence of amino acids that is likely to be highly immunogenic. If possible, choose a sequence of 12–15 amino acids that contains no more than four adjacent hydrophobic residues and no more than six hydrophobic residues in total—the greater the number of charged amino acids in the sequence the better. Peptides with these properties are likely to be soluble in aqueous solvents and therefore easy to couple to carrier proteins. In addition, the cognate sequence is more likely to be present on the surface of the target protein and therefore accessible to the antibody.

Usually, the synthetic peptide is coupled to a carrier protein such as

keyhole limpet hemocyanin (KLH) through an amino- or carboxy-terminal cysteine residue. Ideally, therefore, the sequence of the peptide should end with a "natural" cysteine that is present in the cognate sequence of the target protein. If this is not possible, choose a peptide that contains no cysteine at all. Chemical synthesis of the peptide, which proceeds from the carboxyl terminus to the amino terminus, is then initiated from an "artificial" cysteine that has no counterpart in the cognate sequence of the target protein. This cysteine residue is used as a linker to couple the synthetic peptide to the carrier protein.

COUPLING OF SYNTHETIC PEPTIDES TO KEYHOLE LIMPET HEMOCYANIN

This method, from the laboratory of M. Brown and J. Goldstein (pers. comm.), is a modification of a technique described originally by Green et al. (1982).

1. Dilute KLH with phosphate-buffered saline (pH 6.0) (PBS; see Appendix B) to a final concentration of 10 mg/ml.

   KLH (Calbiochem no. 374817) is supplied at a concentration of 100 mg/ml in 50% glycerol. KLH should be stored at 4°C.

   The pH of PBS is adjusted to pH 6.0 with 3 N HCl.

2. Transfer 1 ml of the KLH solution to a clean 15-ml tube. Put a small, Teflon-covered magnet in the tube, and stir the solution vigorously on a magnetic stirrer. Add 50 μl of dimethyl sulfoxide (DMSO) containing 1.5 mg MBS *under the surface of the KLH solution*. Continue stirring for another 30 minutes.

   MBS is *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Sigma M 8759). It was first used as a coupling agent by Liu et al. (1979).

3. Prepare a column (20-ml bed volume) of Sephadex G-25, equilibrated in PBS (pH 7.4). Apply the sample of KLH to the column and begin to collect 1-ml samples. Immediately after the void volume, a cloudy, grayish material elutes from the column; this is KLH-MBS.

   The purpose of the column is to remove unconjugated MBS, DMSO, and glycerol from the sample.

   If necessary, the elution profile of KLH-MBS can be monitored by measuring the OD_{280} of the column effluent. The recovery of KLH-MBS is usually > 80%.

4. Dissolve 5 mg of the synthetic peptide in the smallest possible volume of PBS (pH 7.4). This step is best carried out as follows:

   a. Add 5 mg of the peptide to 200 μl of PBS in a 15-ml tube. Continue to add PBS in 100-μl aliquots until the peptide is completely dissolved or the volume of the solution is 500 μl.

   b. If the peptide is not dissolved, add 5 N NaOH in 5-μl portions until a total of 25 μl has been added.

   c. If the peptide is still not dissolved, add DMSO in 100-μl portions until a total of 500 μl has been added.

5. Mix the KLH-MBS solution from step 3 with the dissolved peptide. *Vortex vigorously to mix the two solutions.* Stir the mixture for 1 hour at room temperature.

6. Store the solution at −20°C until it is needed for injection into animals.

Notes

i. If the peptide is too hydrophobic, it will not dissolve, and alternative, less efficient ways must then be used to raise antiserum. For example, the hydrophobic peptide can be emulsified with Freund's complete adjuvant and injected without solubilization. This results in the production of specific antibodies that are often of low titer and avidity.

ii. Upon thawing, the cross-linked peptide may form a precipitate. If so, break up the aggregates by sonication or by passage through a 22-gauge hypodermic needle. For immunization, mix approximately 300 μg of cross-linked peptide with an equal volume of Freund's complete adjuvant and inject the emulsion into mice (subcutaneously) or rabbits (intramuscularly or intradermally) as described on pages 18.5–18.6. Two weeks later, boost the animals with a second injection. Mice should be boosted again, 1 week later, with an intraperitoneal injection of 200 μg of peptide mixed with 4 mg of alum. Rabbits should be boosted every 6 weeks with 200 μg of peptide mixed with 4 mg of alum. Mice should be bled 4–5 weeks after the initial injection; rabbits should be bled 1–2 weeks after the second booster injection and at regular intervals thereafter.

Collection and Storage of Antisera

Blood from animals that have been fasted for several hours should be collected in sterile centrifuge tubes or bottles (without citrate or heparin) and allowed to clot at room temperature. A glass rod or sealed pasteur pipette should then be used to "ring" the clot (i.e., to loosen the clot from the wall of the tube or bottle). During the next several hours, the clot will retract to about one half of its original volume, leaving the antiserum as a straw-colored liquid. Transfer as much as possible of the antiserum to a fresh tube, and then centrifuge the clot at 1500*g* for 10 minutes at room temperature. Combine the supernatant with the previously removed antiserum, discard the clot, and store the antiserum.

Antisera may be stored as a lyophilized powder at room temperature, as a solution frozen at −20°C or −70°C, or at 4°C in the presence of 0.02% sodium azide. Although lyophilization is preferred on theoretical grounds, it is impractical for many laboratories and unnecessary for most purposes. Some guidelines for storing antisera are listed below.

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

1. Antisera should be stored in small aliquots that are labeled with the name of the animal and the date of the bleed. Polyclonal antisera are best stored undiluted, whereas monoclonal antibodies should be stored at concentrations of >1 mg/ml in solutions containing 0.1 M NaCl.
2. Do not mix antisera taken from different animals or from different bleeds taken from the same animal. Individual sera can contain cross-reactive antibodies and, during a course of immunizations, an individual animal may respond to other environmental antigens that also are cross-reactive. To avoid the possibility of adulterating a batch of good antiserum with one that is less desirable, keep each bleed from each animal separate.
3. Antisera stored at −20°C are stable for several years. However, repeated freezing and thawing of antisera causes denaturation and aggregation of immunoglobulins, with a consequent decrease in potency. Once a frozen aliquot is thawed, it should thereafter be stored at 4°C.
4. Immunoglobulins of the IgM class are far less robust than IgG and tend to denature when stored at −20°C, which is near the eutectic point of NaCl solutions. Antisera that contain high levels of IgM directed against the target antigen should therefore be stored lyophilized or at −70°C.
5. Antisera gradually become turbid during prolonged storage at 4°C. The precipitate, which is composed mostly of lipoproteins and other cryoproteins whose solubility is limited at 4°C, does not affect the ability of the immunoglobulins to react with their target antigens. Antisera drawn from animals that have not been fasted contain higher levels of lipoprotein and rapidly become turbid during storage at 4°C. Do not mistake this turbidity for bacterial growth. Before use, antibodies should be centrifuged briefly (at 12,000*g* for 2 minutes at 4°C in a microfuge) to sediment any particulate matter and to cause lipoproteins to float to the surface.

Purification of Antibodies

For most purposes, antisera need not be fractionated before use. However, if the antisera are to be radiolabeled or conjugated to enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase, it is necessary to purify the IgG fraction or, in some cases, to purify the antibody of interest by binding it to its cognate antigen.

Although many techniques have been developed to purify IgG molecules, the method of choice is adsorption to, and elution from, beads coated with protein A, a component of the cell wall of *S. aureus* (Hjelm et al. 1972). For reasons that are not known, this protein (M_r = 42,000) binds strongly to sites in the second and third constant regions of the Fc portion of the immunoglobulin heavy chain (Deisenhofer 1981). Each IgG molecule therefore contains two binding sites for protein A. Because protein A itself has four potential sites for binding to IgG (Sjödahl 1977), it is possible to form multimeric complexes of the two types of protein.

Not all immunoglobulins bind to protein A with the same affinity. Antibodies from humans, rabbits, and guinea pigs bind most tightly, followed in decreasing order of affinity by those from pigs, mice, horses, and cows (Kronvall et al. 1970; Goudswaard et al. 1978). Immunoglobulins from goats, rats, chickens, and hamsters bind in a much weaker fashion, and a "bridging" antibody is usually required to purify them by adsorption to protein A. Within any one species, different classes of immunoglobulins vary in the sequences of their Fc regions and consequently bind to protein A with different affinities. Of the major classes of human IgG, for example, three (IgG1, 2, and 4) bind with high affinity and one (IgG3) binds very weakly, if at all. Similarly, mouse IgG2a binds with high affinity, IgG2b and IgG3 bind tolerably well, and IgG1 binds poorly (Ey et al. 1978). These differences are generally unimportant when dealing with polyclonal sera, where antibodies against the target antigen are distributed throughout all of the major subclasses of IgG. Consequently, purification of polyclonal immunoglobulins raised in rabbits, humans, and mice by binding to protein A may alter the distribution of subclasses of IgG, but it rarely changes the specificity or avidity of the final preparation. However, monoclonal antibodies secreted from hybridomas carry only one subclass of heavy chain. Before attempting to purify a given monoclonal antibody, it is essential to determine the subclass of its heavy chain, using commercially available immunological reagents directed against isotypes of the Fc region. If the monoclonal antibody falls into a class that binds poorly to protein A (e.g., human IgG3 or mouse IgG1), it should be purified by another method (e.g., ammonium sulfate precipitation followed by chromatography on DEAE-cellulose). Alternatively, a bridging antibody can be used to attach the monoclonal antibody to protein A.

PURIFICATION OF ANTIBODIES BY ADSORPTION TO PROTEIN A

Protein A coupled to a solid support by cyanogen bromide is supplied by several manufacturers (e.g., protein A–Sepharose CL-4B; Pharmacia). Each milliliter of swollen gel can bind approximately 10–20 mg of IgG (equivalent to 1–2 ml of antiserum). Antibodies bind to protein A chiefly by hydrophobic interactions (Deisenhofer 1981) that can be disrupted at low pH. Protein A is remarkably resilient and withstands repeated cycles of exposure to low pH extremely well; it can also be treated with high concentrations of denaturing agents such as urea, guanidine hydrochloride, or potassium isothiocyanate without permanent damage. Most antibodies can withstand transient exposure to low pH, and this is now the standard method to release them in an active form from protein A–Sepharose beads.

The following is a modification of the method of Goudswaard et al. (1978).

1. Prepare a column of protein A–Sepharose, equilibrated in 100 mM Tris · Cl (pH 8.0) according to the manufacturer's instructions. Each milliliter of swollen gel can adsorb between 10 mg and 20 mg of pure IgG.

   Protein A–Sepharose is very expensive and should be used sparingly. For the preparation of IgG for most laboratory purposes, 1-ml columns are poured and run in pasteur pipettes plugged with sterile glass wool.

2. Add 0.1 volume of 1 M Tris · Cl (pH 8.0) to the antibody preparation (serum or monoclonal antibodies derived from hybridomas grown either in tissue culture or in the peritoneal cavity of mice).

   The concentration of IgG in serum obtained from healthy animals is approximately 10 mg/ml. However, the concentration of antibody in supernatants of hybridomas or in ascitic fluid is highly variable, ranging from 10 μg/ml to 100 μg/ml in the former case and from 1 mg/ml to 20 mg/ml in the latter. To determine the concentration of antibody in serum, analyze an aliquot by SDS-polyacrylamide gel electrophoresis and compare the intensity of staining of the heavy and light chains with a set of immunoglobulin standards run on the same gel. When the antibody is the dominant protein in the solution (e.g., in ascitic fluid or in the supernatant of efficiently secreting hybridomas), its approximate concentration can be estimated from the OD_{280} of the solution (1 OD_{280} = 0.75 mg/ml of pure IgG).

3. Load the antibody preparation on the column, and then wash the column with 10 column volumes of 100 mM Tris · Cl (pH 8.0) followed by 10 volumes of 10 mM Tris · Cl (pH 8.0).

4. Add 1 column volume of 100 mM glycine (pH 3.0). Begin collecting 500-μl fractions of the column eluate in microfuge tubes containing 50 μl of 1 M Tris · Cl (pH 8.0).

5. Just as the column runs dry, add another 1 column volume of 100 mM glycine (pH 3.0) and continue collecting samples. Continue this process until 5 column volumes have eluted from the column.

6. Identify the fractions that contain IgG by measuring their absorbance at 280 nm, using elution buffer as a blank. The concentration of IgG in the column eluate should be at least 1 mg/ml (an OD_{280} of 1.33).

7. Dispense the IgG into aliquots and store them as described on page 18.10.

8. Remove residual proteins from the column by washing it sequentially with 10 volumes of 3 M urea, 1 M LiCl, and 100 mM glycine (pH 2.5). Finally, readjust the pH of the column to 8.0 by washing it with 10 volumes of 100 mM Tris · Cl (pH 8.0). Store the column at 4°C in 100 mM Tris · Cl (pH 8.0) containing 0.02% sodium azide.

   Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

PURIFICATION OF ANTIBODIES BY ADSORPTION

Although hyperimmune antisera raised in experimental animals contain very high concentrations of immunoglobulin directed against the target antigen, such antisera also always contain antibodies directed against other antigens. In addition, the immunoglobulins in antisera may bind with low avidity to molecules that are not true target antigens. For these and other reasons, antisera can manifest a level of background reactivity that is unacceptably high. There are three ways to deal with this problem:

- An innocuous blocking agent (e.g., bovine serum albumin or normal serum) can be used to compete with the immunoglobulin for nonspecific binding sites.
- Antibodies that are directed against specific contaminating antigens (e.g., bacterial antigens) can be removed by adsorption.
- Antibodies directed against the target antigen can be separated from contaminating antibodies by affinity purification.

Blocking agents are routinely included in solutions used, for example, in immunological screening of expression libraries constructed in plasmid or bacteriophage λ vectors (see Chapter 12). Methods to remove antibacterial antibodies from antisera used for such screening are described on pages 12.25–12.28. In the section that follows, we describe techniques to remove antibodies that cross-react with components in eukaryotic cells and discuss methods that are available for affinity purification of antibodies.

Removal of Cross-reacting Antibodies from Antisera

To remove antibodies that react with antigens present in mammalian cells, use an acetone extract of a cell line or tissue that is known not to express the true target antigen. If such a cell line is not available or cannot be identified with certainty, use an acetone extract of commercially available dried yeast.

1. Resuspend the tissue or cells in 0.1 M NaCl, using 2 ml of ice-cold saline solution for every gram of tissue or cells. The suspension should be as homogeneous as possible, with no fibrous material or large clumps of cells. If necessary, filter the tissue suspension through three layers of cheesecloth.

2. Add 4 volumes of acetone, chilled to −20°C, and mix the suspension vigorously. Store the suspension on ice for 1 hour.

3. Centrifuge the suspension at 10,000*g* for 10 minutes at 4°C. Discard the supernatant, and resuspend the pellet in acetone (−20°C) using the same amount that was used in step 2. Vigorous vortexing may be necessary to achieve complete resuspension. Store the suspension on ice for 10 minutes.

4. Recentrifuge, and discard the supernatant. Transfer the pellet to a piece of Saran Wrap. Allow the acetone to evaporate in a chemical hood. Mix the pellet from time to time to speed evaporation.

5. When the powder is completely dry, transfer it to a container with a tight-fitting lid. Store the powder at −20°C until needed. Each gram of tissue or cells yields approximately 100 mg of powder.

6. To remove cross-reacting antibodies from antiserum, add the powder to a final concentration of 1% (w/v). Mix the suspension carefully, taking care to avoid producing bubbles and froth, which denature proteins. Store the suspension on ice for 15 minutes. Remove the powder by centrifugation at 10,000*g* for 10 minutes at 4°C. Store the supernatant, which should now be free of cross-reacting antibodies, in small aliquots at −20°C.

Purification of Immunospecific Antibodies

Antibodies may be purified by adsorption to, and elution from, their cognate antigen. In some cases (e.g., when the antigen is a protein), the antigen may be coupled to a matrix such as cyanogen-bromide-activated Sepharose. Antibodies directed against epitopes displayed by the protein will be retained by the column; all other immunoglobulins will pass through. The bound antibody is then released from the column by agents that disrupt the antigen-antibody complex (e.g., potassium thiocyanate, low-pH buffers, etc.). Details of the methods used to prepare antibodies by immunochromatography vary from antigen to antigen and from antibody to antibody. However, the general principles are well-described in a number of reviews (see, e.g., Hurn and Chantler 1980; Harlow and Lane 1988). When using these methods, it is essential to use highly purified antigen and to avoid the batch of antigen that was used to raise the antibody in the experimental animals. Furthermore, it is important to remember that antibodies with different affinities for the antigen will show different patterns of elution from the column; those that bind loosely to the antigen will elute first, and those that bind most tightly will elute last. In fact, antibodies with the highest avidity may be denatured by the elution buffer before they dissociate from the antigen. Thus, there is a tendency during immunopurification to select for antibodies that are specific for the antigen but that bind with low affinity.

Antibodies may be purified on a small scale by adsorption to, and elution from, protein antigens that are immobilized on diazotized paper (Olmsted 1981) or nitrocellulose filters (Burke et al. 1982; Smith and Fisher 1984; Earnshaw and Rothfield 1985) after electrophoresis through SDS-polyacrylamide gels. Antibodies prepared by this method are especially useful for confirming the identity of cDNA clones isolated from expression libraries constructed in bacteriophage λ or plasmid expression vectors. For example, many false-positive clones can be eliminated by purifying antibodies from crude sera by virtue of their ability to bind to a fusion protein partly encoded by the cloned cDNA and testing the ability of these purified antibodies to precipitate the target protein or to react with it on a western blot. However, this method works well only when the antibodies react with epitopes that are displayed on denatured proteins. Typically, about 50 ng of immunopurified antibody are recovered per microgram of target protein loaded on the original SDS-polyacrylamide gel. Because of the idiosyncratic nature of the interactions between antibodies and their target proteins, it is not possible to give conditions for binding and elution that are universally applicable. For example, most antibodies can be eluted from their immobilized antigens with glycine buffer (pH 2.8). However, Earnshaw and Rothfield (1985) found that antibodies to human centromeric proteins could be eluted only with a solution containing 3 M potassium thiocyanate and 0.5 M NH_4OH. Anyone who wishes to use this powerful technique should therefore be prepared to invest some effort in defining the optimal conditions for binding and release of their particular antibodies from their target proteins. However, the following protocol, which is a modification of Olmsted's (1981) method produced by J. Allan and M. Douglas (pers. comm.), has worked well with a number of rabbit polyclonal antibodies directed against a variety of mammalian and yeast proteins.

AFFINITY PURIFICATION OF MONOSPECIFIC ANTIBODIES USING ANTIGEN IMMOBILIZED ON NITROCELLULOSE FILTERS

1. Load a preparation containing the target antigen along the entire length of an SDS-polyacrylamide gel (see pages 18.47–18.54). Between 650 μg and 1000 μg of soluble protein can be loaded on a gel of standard size without overloading. After the gel has been run, transfer the proteins from the gel to a nitrocellulose filter as described on pages 18.64–18.66.

2. After transfer, incubate the filter in blocking buffer for 1 hour at room temperature. Use 0.25 ml of blocking buffer for each square centimeter of nitrocellulose filter.

   Blocking buffer

   3% bovine serum albumin in phosphate-buffered saline (PBS; see Appendix B) containing 0.02% sodium azide.

   Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

   The bovine serum albumin serves as a blocking agent by binding to sites on the nitrocellulose filter that are not occupied by proteins transferred from the gel.

3. Add the first antibody, and incubate for 5 hours at room temperature or for 16 hours at 4°C with gentle shaking.

   The first antibody, which should react strongly with the denatured target antigen, can usually be diluted 500- to 1000-fold in the blocking buffer.

4. Discard the antibody solution, and rinse the nitrocellulose filter in 0.15 M NaCl for 20 minutes at room temperature.

5. Rinse the filter in PBS for 20 minutes at room temperature.

6. Using a sharp scalpel blade, cut a strip approximately 0.2 cm wide from each side of the filter. Transfer the strips to a petri dish, and stain them with horseradish-peroxidase (HRP)-coupled antibody directed against the first antibody (see page 18.75). Meanwhile, continue washing the main section of the nitrocellulose filter in PBS.

   HRP-coupled second antibody should be used at the dilutions recommended by the manufacturer.

7. Remove the stained strips from the petri dish and align them with the main section of the filter. Using a soft-lead pencil, mark the position(s) of the band(s) of interest. Cut out strips of nitrocellulose filter that carry the target antigen.

8. Arrange the nitrocellulose strips on pieces of Parafilm that are pressed onto the bottoms of petri dishes. Add a small volume of elution buffer to each strip (200–500 μl, depending on the width of the strip). Incubate the strips on a rocking platform in a humidified atmosphere for 20 minutes at room temperature.

The Parafilm prevents the elution buffer from spreading over the surface of the dish. A typical humidified tissue culture incubator set at room temperature provides an adequate moist atmosphere.

Elution buffer
0.2 M glycine (pH 2.8)
1 mM EGTA

9. Transfer the eluted antibody to a microfuge tube. Neutralize the elution buffer as soon as possible by adding 0.1 volume of 1 M Tris base. Check that the pH is near neutrality by spotting an aliquot of the solution on pH paper.

10. Add 0.1 volume of 10× PBS. Add sodium azide to a final concentration of 0.02%, and store the antibody at 4°C.

Notes

i. The strip of nitrocellulose can be used several times in succession to purify antibody specific for the immobilized antigen. However, the strip should not be allowed to dry between successive rounds of purification and must be retreated with blocking buffer after each round of elution.

ii. Antibodies of the appropriate subclass purified in this way may be concentrated by adsorption to, and elution from, protein A–Sepharose.

Immunological Assays

Antibodies are used in a wide variety of assays, both qualitative and quantitative, to detect and measure the amount of target antigens. These assays include immunoprecipitation, western blotting, and solid-phase radioimmunoassays (RIAs), which will be described in detail in the remainder of this chapter.

SOLID-PHASE RADIOIMMUNOASSAY

The solid-phase RIA is a quantitative method that is capable of detecting as little as 1 pg of target antigen. This means that RIAs are sufficiently sensitive to measure, for example, the amount of foreign protein produced by transfected mammalian cell cultures. There are many different kinds of RIAs, which fall into four basic designs:

- *Competition RIAs:* In this method, the unlabeled target protein in the test sample competes with a constant amount of radiolabeled protein for binding sites on the antibody. The amount of radioactivity present in the unbound or bound target protein is then measured. This type of assay can be extremely sensitive but requires that target protein is available (preferably in a pure form) to serve both as a competitor and as a standard.
- *Immobilized antigen RIAs:* In this method, unlabeled antigen is attached to a solid support and exposed to radiolabeled antibody. Comparison of the amount of radioactivity that binds specifically to the samples under test with the amount that binds to a known amount of immobilized antigen allows the antigen in the test samples to be quantitated. Although used occasionally, this type of assay is not particularly useful for quantitation of small amounts of foreign protein in complex mixtures (e.g., in cell lysates); most of the binding sites on the solid support become occupied by proteins other than the target protein, so that the sensitivity of the assay is comparatively low.
- *Immobilized antibody RIAs:* In this method, a single antibody bound to a solid support is exposed to radiolabeled antigen. The amount of antigen in the test sample can be determined by the amount of radioactivity that binds to the antibody. This assay is not useful for quantitating the amount of foreign protein in many different samples, chiefly because of the practical difficulty of radiolabeling the protein either in vivo or in vitro.
- *Double-antibody RIAs:* In this method, one antibody bound to a solid support is exposed to the unlabeled target protein. After washing, the target protein bound to the immobilized antibody is quantitated with an excess of a second radiolabeled antibody. This assay is extremely sensitive and specific because the target protein is essentially purified and concentrated by immunoadsorption. Furthermore, many test samples can be processed simultaneously. However, the method requires that the first and

second antibodies recognize nonoverlapping epitopes on the target protein. Ideally, the first antibody is monoclonal, whereas the second can be either a polyclonal antibody or a monoclonal antibody of different specificity. However, in some cases, it may be possible to use the same polyclonal antibody for both parts of the assay. If suitable antibodies are available, this is the method of choice for quantitation of target proteins in complex mixtures.

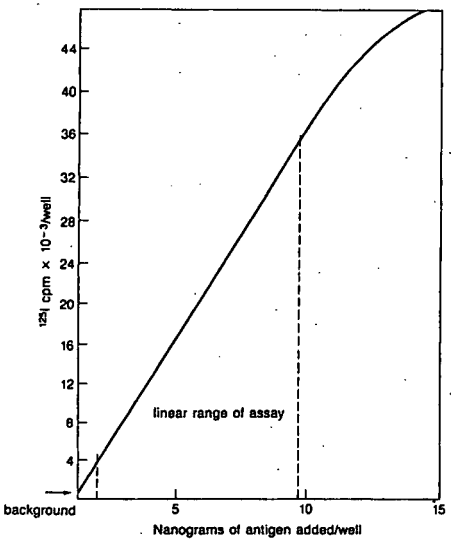

FIGURE 18.1

Solid-phase Radioimmunoassays Using Two Antibodies

The main features that affect the quality of the results obtained with RIAs are the sensitivity, accuracy, and specificity of the assay.

The sensitivity of the assay is determined by two factors:

- *The amount of the first antibody that is bound to the solid support (usually a 96-well polyvinyl chloride [PVC] plate).* There are a finite number of binding sites on the surface of the solid support and, at saturation, each well of the plate binds approximately 100 ng of pure IgG ($\sim 6 \times 10^{11}$ molecules). However, antibodies directed against the target protein must compete with immunoglobulins and other components in the serum for binding sites on the solid support. To maximize binding of relevant antibodies, immunoglobulins directed against the target protein should first be purified by immunoaffinity chromatography. Frequently, however, excellent results may be obtained using immunoglobulins purified from hyperimmune sera either by adsorption to, and elution from, protein A–Sepharose or by chromatography on DEAE-cellulose.
- *The specific activity of the second radiolabeled antibody.* Antibodies radiolabeled with ^{125}I as described later in this chapter (see pages 18.24–18.25) have an approximate specific activity of 5×10^6 cpm/μg. The amount of the radiolabeled second antibody that reacts with antigen-antibody complexes bound to a solid support varies from antigen to antigen. Typically, however, a saturating amount of protein antigen attached to an immobilized first antibody might be expected to bind approximately 2×10^4–1×10^5 cpm of radiolabeled second antibody per well.

The accuracy of the assay is determined by the concentration of antigen in solution, which should not be so high as to saturate the antibody bound to the solid support. Quantitative results can be obtained only when the amount of antigen bound to the first antibody is proportional to its concentration in the sample being tested. To compare the amount of antigen in a number of test samples, assay several different dilutions of each sample (blocking buffer should be used as a diluent). Plot the amount of radioactive second antibody bound to each well against the dilution of the sample. Within the linear range of the assay (see Figure 18.1), there is a proportional relationship between the amount of radioactivity that is specifically bound and the dilution of antigen. In some cases, it may be possible to calculate the absolute amount of antigen present in a test sample from a standard curve obtained using known amounts of pure antigen. If a standard curve with known amounts of pure antigen is not available, the relative amounts of antigen in different samples may be estimated by calculating the dilution of antigen that will give half-maximal binding of radiolabeled antibody. In this case, it is essential that the dilutions span a wide range from background to maximal binding. For accurate quantitation of antigen, it is essential that the radiolabeled second antibody be present in excess. The appropriate amount of second antibody should be determined in pilot experiments in which different amounts of radiolabeled antibody are added to saturating amounts of bound antigen-antibody complexes.

The specificity of the assay is determined chiefly by the quality of the antisera used. However, even when monoclonal antibodies or immuno-

globulins purified from hyperimmune sera are used, there is always the chance of immunological cross-reactions. For this reason, it is essential to include the appropriate controls every time a solid-phase RIA is carried out. For example, when measuring the amount of a foreign protein synthesized by a transfected culture of mammalian cells, it is necessary to use extracts of mock-transfected cells as negative controls. Other essential controls include:

- *Immunoglobulins purified from preimmune serum,* which should be used in some wells in place of antibodies directed against the target antigen. If a monoclonal antibody is used as the first antibody, use a monoclonal antibody directed against an irrelevant antigen as a control. The amount of radiolabeled antibody that binds to wells treated with preimmune immunoglobulin and saturating amounts of antigen should be at least tenfold lower than the amount that binds to wells exposed to immune immunoglobulin and antigen. If the immune serum is highly specific, the signal-to-noise ratio can be as high as 100:1 in solid-phase RIAs.

- *Blank wells,* which are exposed to phosphate-buffered saline (PBS; see Appendix B) instead of first antibody. The blank wells are then exposed to blocking buffer and radiolabeled second antibody as described below. If blocking is complete, negligible amounts of radiolabeled second antibody should bind to the blank wells.

- *Positive wells,* which are exposed to known quantities of antigen.

A generalized protocol for a solid-phase RIA is given below.

1. Plan the experiment, and draw a key so that you will know what each well on the plate contains.

2. To each well of a PVC plate, add 50 μl of IgG solution (20 μg/ml in 0.2 M $NaHCO_3$). Cover the plate, and incubate it for 2 hours at room temperature in a humidified atmosphere.

   A typical humidified tissue culture incubator set at room temperature provides an adequate moist atmosphere.

   The covers supplied with the plates fit very loosely, and incubation in a humidified atmosphere is necessary to prevent drying of the samples at the edges of the plates. An alternative way to deal with this problem is to purchase plates without covers (which are less expensive) and to wrap them tightly in Saran Wrap. The plates can then be incubated in the open on the bench.

3. Aspirate the IgG solution from the wells. The solution may be saved, stored at 4°C, and reused several times.
   Important: Do not let the wells become dry at any stage during the remainder of the protocol.

4. Using a squirt bottle, wash the wells twice with blocking buffer. The IgG solution and the washes can be removed quickly and efficiently by flicking the plate over the sink or a suitable waste container.

Blocking buffer

3% bovine serum albumin in PBS containing 0.02% sodium azide.

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

5. Fill the wells with blocking buffer. Cover the plate and incubate it for 20 minutes at room temperature in a humidified atmosphere.

   The bovine serum albumin serves as a blocking agent by binding to sites on the PVC plate that are not occupied by immunoglobulin molecules.

   The antibody-coated plates may be stored for several days at 4°C if the wells are completely filled with blocking buffer. Before use, plates that have been stored in this fashion should be rinsed with blocking buffer.

6. Wash the wells twice more with blocking buffer (see step 4).

7. Make a series of dilutions in blocking buffer of the antigen that is to be assayed. Add 50 μl of each dilution to each of two wells. Cover the plate and incubate it for 2 hours at room temperature in a humidified atmosphere or for 8 hours at 4°C.

8. Wash the plate four times with blocking buffer (see step 4).

9. Dilute the second antibody, radiolabeled with ^{125}I as described on pages 18.24–18.25, in blocking buffer. To each well, add 50 μl of radiolabeled antibody containing 50,000–200,000 cpm (5–20 ng). Cover the plate and incubate it for at least 2 hours at room temperature in a humidified atmosphere.

10. Wash the plate several times with blocking buffer. Quickly remove as much fluid as possible from each well by aspiration, using a pasteur pipette attached to a vacuum line equipped with traps (see Appendix E, Figure E.1).

11. Using sharp scissors, cut individual wells from the plate. Place each well in a labeled counting vial, and measure the radioactivity in a gamma counter.

12. Plot the amount of radioactivity specifically bound to the test samples. Calculate the dilution of each sample that yields half-maximal binding of radiolabeled antibody. Calculate the amount of antigen present in each sample relative to the controls.

    The amount of specifically bound radioactivity varies considerably from antigen to antigen and from antiserum to antiserum. However, it would be reasonable to expect 10 ng of purified, immobilized target antigen to bind between 1000 and 10,000 cpm of radioiodinated antibody.

Iodination of Antibodies

Of the several methods that are available to radioiodinate antibodies, the most commonly used is a reaction in which $H_2O^{125}I^+$, generated by oxidation of $Na^{125}I$ with chloramine-T, attacks the side chains of tyrosine residues and, to a lesser extent, histidine residues (Greenwood et al. 1963; McConahey and Dixon 1966, 1980). This modification, if not carried out to excess, generally does not affect the ability of the antibody to interact with its target antigen. However, in some cases (presumably when the antibody has a tyrosine residue in its antigen binding site), the immunoreactivity of radioiodinated antibody may be significantly reduced. It is then necessary to use a conjugation reagent such as Bolton-Hunter reagent (Bolton and Hunter 1973). The *N*-succinimidyl group of this reagent (*N*-succinimidyl 3-[4-hydroxy 5-[^{125}I]iodophenyl]propionate) condenses with the free amino and imino in lysine and histidine residues to give a derivative in which the radioiodinated phenyl group is linked to an amide bond in the target protein. (For methods involving this reagent, see Langone 1980; Harlow and Lane 1988.)

Caution: ^{125}I is a potential health hazard. It emits low-energy γ- and X-rays, is volatile, and accumulates in the thyroid. Under United States regulations, the maximum allowable load of ^{125}I in the thyroid is 1.2 μCi. However, the procedures established by the local radiation safety committee, if strictly followed, should ensure that those working with ^{125}I stay well within this limit.

RADIOIODINATION OF ANTIBODIES USING THE CHLORAMINE-T METHOD

1. Equilibrate Sephadex G-25 in phosphate-buffered saline (PBS; see Appendix B) according to the manufacturer's instructions. Prepare a column in a pasteur pipette that is plugged with sterile glass wool. Run at least 10 column volumes of PBS containing 1% bovine serum albumin through the column. Then wash the column with 5 volumes of PBS lacking bovine serum albumin. When the column is ready, wrap a piece of Parafilm around the bottom of the pipette. This prevents the column from running dry.

   Prepared columns (PD-10 Sephadex G-25M) can be purchased from Pharmacia.

   Pretreatment with bovine serum albumin reduces nonspecific adsorption of antibody to the column and resin. It is important to remove bovine serum albumin from the column buffer before applying the radioiodinated antibody; otherwise, there is a possibility that the bovine serum albumin in the solution will become radioiodinated.

2. In a microfuge tube, add up to 70 μg of antibody (dissolved in 10 μl of PBS) to 50 μl of 50 mM sodium phosphate buffer (pH 7.2).

   Since the radioiodination of proteins involves oxidation of radioiodide by chloramine-T, it is essential that no reducing agents be present in any of the solutions used.

   Caution: The following steps must be carried out in a ventilated chemical hood behind shielding that is sufficient to protect the worker from direct radiation. Consult your radiation safety officer before beginning work with ^{125}I, and make sure you are familiar with the procedures used to monitor and dispose of the radioisotope.

3. Add 500 μCi of carrier-free $Na^{125}I$ and mix the solution well.

   $Na^{125}I$ is usually supplied in 0.1 N NaOH, which minimizes oxidation. 50 μl of sodium phosphate buffer can accommodate up to 7 μl of 0.1 N NaOH without significant change in pH. However, the sudden addition of larger amounts of NaOH to the antibody can cause denaturation. This can be avoided by neutralizing the radioiodide solution with an equal volume of 0.1 N HCl just before use. Neutralized solutions of $Na^{125}I$ must be used immediately to avoid loss of the volatile ^{125}I. Carrier-free $Na^{125}I$ should be used to minimize the total amount of iodine incorporated into the antibody.

4. Add 15 μl of a freshly prepared solution of chloramine-T (2 mg/ml in 0.1 M sodium phosphate buffer [pH 7.2]).

5. Incubate the mixture for 60 seconds at room temperature.

6. Add 50 μl of stop buffer, and immediately apply the mixture to the column of Sephadex G-25.

   Stop buffer

   0.1 M sodium phosphate buffer (pH 7.2) containing
   10 mg/ml tyrosine
   2 mg/ml sodium metabisulfite
   10% glycerol
   0.1% xylene cyanol FF

7. Begin collecting 100–150-μl fractions of the eluate from the column. After the sample has entered the resin, add PBS (lacking bovine serum albumin) to the top of the column. Make sure that the column does not run dry at any stage.

8. Using a hand-held minimonitor, check the radioactivity that elutes from the column. If the reaction has gone well, approximately 90% of the radiolabel should be incorporated into the antibody. The radiolabeled antibody should elute well ahead of the xylene cyanol FF, which runs with the unincorporated radiolabel. When the antibody has eluted, seal the bottom of the pipette with modeling clay. Dispose of the entire column, which still contains unincorporated radiolabel, in the radioactive waste.

9. Combine the fractions containing the radiolabeled antibody. Add bovine serum albumin to a final concentration of 1%. Measure the radioactivity in a 5-μl aliquot, and store the antibody at 4°C until it is required for use.

   If the immunoreactivity of the antibody is reduced by the above procedure, test the effects of metabisulfite on the protein. If metabisulfite damages the protein, omit it from the stop buffer. If, as is most likely, the antibody is insensitive to metabisulfite, the reduction in immunoreactivity must be caused by exposure to chloramine-T or to the incorporation of iodine into sensitive tyrosine residues. In either case, it will be necessary to try another method of radioiodination, for example, Bolton-Hunter reagent (Langone 1980; Harlow and Lane 1988).

   The specific activity of the radiolabeled antibody should be $\sim 5 \times 10^6$ cpm/μg.

TRANSFER OF PROTEINS FROM SDS-POLYACRYLAMIDE GELS TO SOLID SUPPORTS: IMMUNOLOGICAL DETECTION OF IMMOBILIZED PROTEINS (WESTERN BLOTTING)

Western blotting (Towbin et al. 1979; Burnette 1981) is to proteins what Southern blotting is to DNA. In both techniques, electrophoretically separated components are transferred from a gel to a solid support and probed with reagents that are specific for particular sequences of amino acids (western blotting) or nucleotides (Southern hybridization). In the case of proteins, the probes usually are antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the solid support. Western blotting is therefore extremely useful for the identification and quantitation of specific proteins in complex mixtures of proteins that are not radiolabeled. The technique is almost as sensitive as standard solid-phase radioimmunoassays and, unlike immunoprecipitation, does not require that the target protein be radiolabeled. Furthermore, because electrophoretic separation of proteins is almost always carried out under denaturing conditions, any problems of solubilization, aggregation, and coprecipitation of the target protein with adventitious proteins are eliminated.

The critical difference between Southern and western blotting lies in the nature of the probes. Whereas nucleic acid probes hybridize with a specificity and rate that can be predicted by simple equations (see Chapter 10), antibodies behave in a much more idiosyncratic manner. As discussed earlier in this chapter, an individual immunoglobulin may preferentially recognize a particular conformation of its target epitope (e.g., denatured or native). Consequently, not all monoclonal antibodies are suitable for use as probes in western blots, where the target proteins are thoroughly denatured. Polyclonal antisera, on the other hand, are undefined mixtures of individual immunoglobulins, whose specificity, affinity, and concentration are often unknown. Consequently, it is not possible to predict the efficiency with which a given polyclonal antiserum will detect different antigenic epitopes of an immobilized, denatured target protein.

Although there is an obvious danger that comes from using undefined reagents to assay a target protein that may also be poorly characterized, most problems that arise with western blotting in practice can be solved by designing adequate controls. These include the use of (1) antibodies (i.e., preimmune sera or irrelevant monoclonal antibodies) that should not react with the target protein and (2) control preparations that either contain known amounts of target antigen or lack it altogether.

Often, there is little choice of immunological reagents for western blotting—it is simply necessary to work with whatever antibodies are at hand. However, if a choice is available, either a high-titer polyclonal antiserum or a mixture of monoclonal antibodies raised against the denatured protein should be used. Reliance on a single monoclonal antibody is hazardous because of the high frequency of spurious cross-reactions with irrelevant proteins. If, as is usually the case, monoclonal and polyclonal antibodies have been raised against native target protein, it will be necessary to verify that they react with epitopes that either (1) resist denaturation with SDS and reducing agents or (2) are created by such treatment. This can be

done by using denatured target antigen in a solid-phase radioimmunoassay (see pages 18.19–18.23) or in western dot blots.

In western blotting, the samples to be assayed are solubilized with detergents and reducing agents, separated by SDS-polyacrylamide gel electrophoresis, and transferred to a solid support (usually a nitrocellulose filter), which may then be stained. The filter is subsequently exposed to unlabeled antibodies specific for the target protein. Finally, the bound antibody is detected by one of several secondary immunological reagents (^{125}I-labeled protein A or anti-immunoglobulin, or anti-immunoglobulin or protein A coupled to horseradish peroxidase or alkaline phosphatase). As little as 1–5 ng of an average-sized protein can be detected by western blotting.

Preparation and Electrophoresis of Samples

Two methods are used to extract proteins for western blotting from cells: Either the intact cells are dissolved directly in sample buffer or an extract is made as described earlier for samples to be immunoprecipitated (see pages 18.30–18.41). Which of these methods is best in any individual case depends on the type of cells and on the properties of the antigen.

- In general, bacteria expressing the target protein are lysed directly in SDS gel-loading buffer as described on pages 18.40–18.41.
- Yeasts are first lysed by vortexing in the presence of glass beads or enzymatically (see pages 18.35–18.36), and the resulting extracts are then prepared (see pages 18.35–18.39).
- Mammalian tissues are usually dispersed mechanically and then dissolved directly in SDS gel-loading buffer.
- Mammalian cells in tissue culture may be lysed gently with detergents as described on page 18.34, or, alternatively, the protocol on pages 18.62–18.63, in which the cells are lysed directly in SDS gel-loading buffer, may be used if the target antigen is resistant to this type of extraction.

In any case, the samples are analyzed by SDS-polyacrylamide gel electrophoresis as described on pages 18.47–18.54.

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To my teachers

Paul F. Brandwein
Daniel L. Harris
Douglas E. Smith
Elkan R. Blout
Edward M. Purcell

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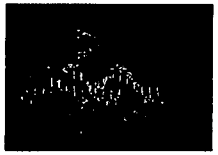


charged terminal amino group of the peptide to form a phenylthiocarbamoyl derivative. Then, under mildly acidic conditions, a cyclic derivative of the terminal amino acid is liberated, which leaves an intact peptide shortened by one amino acid. The cyclic compound is a phenylthiohydantoin (PTH) amino acid, which can be identified by chromatographic procedures. Furthermore, the amino acid composition of the shortened peptide:

(Arg, Asp, Gly_2, Phe)

can be compared with that of the original peptide:

(Ala, Arg, Asp, Gly_2, Phe)

The difference between these analyses is one alanine residue, which shows that alanine is the amino-terminal residue of the original peptide. The Edman procedure can then be repeated on the shortened peptide. The amino acid analysis after the second round of degradation is

(Arg, Asp, Gly, Phe)

showing that the second residue from the amino end is glycine. This conclusion can be confirmed by chromatographic identification of PTH-glycine obtained in the second round of the Edman degradation. Three more rounds of the Edman degradation will reveal the complete sequence of the original peptide.

Analyses of protein structures have been markedly accelerated by the development of *sequenators*, which are automated instruments for the determination of amino acid sequence. In a liquid-phase sequenator, a thin film of protein in a spinning cylindrical cup is subjected to the Edman degradation. The reagents and extracting solvents are passed over the immobilized film of protein, and the released PTH-amino acid is identified by high-performance liquid chromatography (HPLC; Figure 3-19). One cycle of the Edman degradation is carried out in less

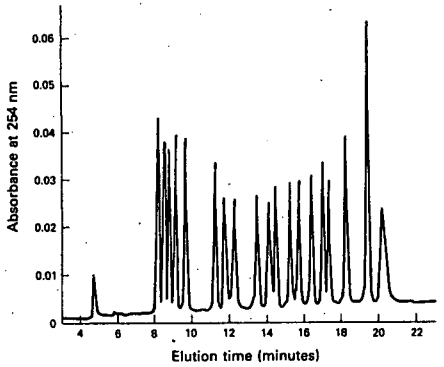


Figure 3-19
PTH-amino acids can be rapidly separated by high-pressure liquid chromatography (HPLC). In this HPLC profile, a mixture of PTH-amino acids is clearly resolved into its components. An individual amino acid can be identified by comparing its profile with this one.

than two hours. By repeated degradations, the amino acid sequence of some fifty residues in a protein can be determined. A recently devised gas-phase sequenator can analyze picomole quantities of peptides and proteins. This high sensitivity makes it feasible to analyze the sequence of a protein sample eluted from a single band of an SDS-polyacrylamide gel.

PROTEINS CAN BE SPECIFICALLY CLEAVED INTO SMALL PEPTIDES TO FACILITATE ANALYSIS

Peptides much longer than about fifty residues cannot be reliably sequenced by the Edman method because not quite all peptides in the reaction mixture release the amino acid derivative at each step. If the efficiency of release of each round were 98%, the proportion of "correct" amino acid released after sixty rounds would be only 0.3 (0.98^{60})—a hopelessly impure mix. This obstacle can be circumvented by specifically cleaving a protein into peptides not much longer than fifty residues. In essence, the strategy is to divide and conquer.

Specific cleavage can be achieved by chemical or enzymatic methods. For example, Bernhard Witkop and Erhard Gross discovered that *cyanogen bromide* (CNBr) splits polypeptide chains only on the carboxyl side of methionine residues (Figure 3-20). A protein that has ten methionines will usually yield eleven peptides on cleavage with CNBr. Highly specific cleavage is also obtained with trypsin, a proteolytic enzyme from pancreatic juice. Trypsin cleaves polypeptide chains on the carboxyl side of arginine and lysine residues (Figure 3-21). A protein

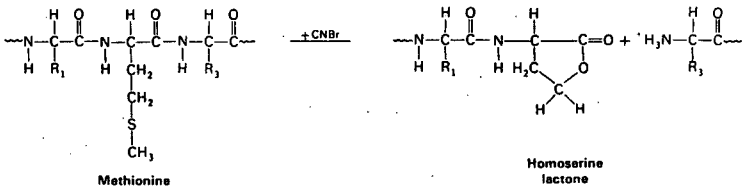


Figure 3-20
Cyanogen bromide cleaves polypeptides on the carboxyl side of methionine residues.

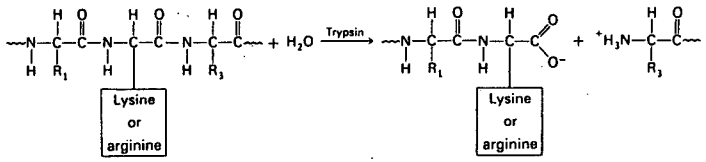
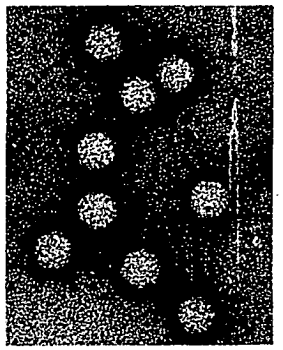
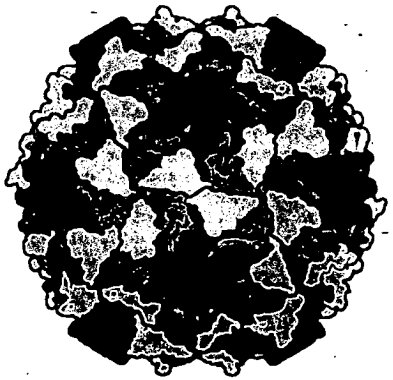


Figure 3-21
Trypsin hydrolyzes polypeptide on the carboxyl side of arginine and lysine residues.



A ⟷ 1000 Å (100 nm)



B

Figure 3-33
Polio virus. (A) Electron micrograph. (B) Model based on x-ray crystallographic analysis. [Part A courtesy of Dr. T. W. Jeng and Dr. Wah Chiu. Part B (computer model and photograph) courtesy of Dr. Arthur J. Olson, Research Institute of Scripps Clinic, © 1987.]

The structures of more than 200 proteins have been elucidated at atomic resolution. Knowledge of their detailed molecular architecture has provided insight into how proteins recognize and bind other molecules, how they function as enzymes, how they fold, and how they evolved. This extraordinarily rich harvest is continuing at a rapid pace and profoundly influencing the entire field of biochemistry. Moreover, x-ray crystallography is being complemented by nuclear magnetic resonance (NMR) spectroscopy, electron microscopy, and electron crystallography, in obtaining increasingly informative views of biomolecules at high resolution. We shall be looking at the three-dimensional structures of proteins and other biomolecules throughout this book and relating the architecture of these molecules to their biological function.

PROTEINS CAN BE QUANTITATED AND LOCALIZED BY HIGHLY SPECIFIC ANTIBODIES

An *antibody* is a protein synthesized by an animal in response to the presence of a foreign substance, called an *antigen* (Chapter 35). Antibodies (also called *immunoglobulins*) have specific affinity for the antigens that elicited their synthesis. Proteins, polysaccharides, and nucleic acids are effective antigens. Antibodies can also be formed to small molecules, such as synthetic peptides, provided that the small molecules are attached to a macromolecular carrier. The group recognized by an antibody is called an *antigenic determinant* (or *epitope*). Animals have a very large repertoire of antibody-producing cells, each producing antibody of a single specificity. An antigen acts by stimulating the proliferation of the small number of cells that were already forming complementary antibody. The major type of antibody in blood plasma is *immunoglobulin G*, a 150-kd protein containing two identical sites for the binding of antigen (Figure 3-34).

Antibodies that recognize a particular protein can be obtained by injecting the protein into a rabbit twice, three weeks apart. Blood is drawn from the immunized rabbit several weeks later and centrifuged. The resulting serum, called an *antiserum*, usually contains the desired antibody. The antiserum or its immunoglobulin G fraction can be used directly. Alternatively, antibody molecules specific for the antigen can be purified by affinity chromatography. Antibodies produced in this way are *polyclonal*—that is, they are products of many different populations of antibody-producing cells and hence differ somewhat in their precise specificity and affinity for the antigen. A major advance of recent years is the discovery of a means of producing *monoclonal antibodies* of virtually any desired specificity (p. 896). Monoclonal antibodies, in contrast with polyclonal ones, are homogeneous because they are synthesized by a population of identical cells (a clone). Each such population is descended from a single *hybridoma cell* formed by fusing an antibody-producing cell with a tumor cell that has the capacity for unlimited proliferation.

Closely related proteins can be distinguished by antibodies; indeed, a difference of just one residue on the surface can be detected. Antibodies can be used as exquisitely specific analytic reagents to quantitate the amount of a protein or other antigen. In a *solid-phase immunoassay*, antibody specific for a protein of interest is attached to a polymeric support such as a sheet of polyvinylchloride (Figure 3-35). A drop of cell extract or a sample of serum or urine is laid on the sheet, which is washed after formation of the antibody-antigen complex. Antibody specific for a different site on the antigen is then added, and the sheet is again washed. This second antibody carries a radioactive or fluorescent label so that it can be detected with high sensitivity. The amount of second antibody bound to the sheet is proportional to the quantity of antigen in the sample. The sensitivity of the assay can be enhanced even further if the second antibody is attached to an enzyme such as alkaline phosphatase. This enzyme can rapidly convert an added colorless substrate into a colored product, or a nonfluorescent substrate into an intensely fluorescent product (Figure 3-36). Less than a nanogram (10^{-9} g) of a protein can readily be measured by such an *enzyme-linked immunosorbent assay* (ELISA), which is rapid and convenient. For example, pregnancy can be detected within a few days after conception by immunoassaying urine for the presence of human chorionic gonadotropin (hCG), a 37-kd protein hormone produced by the placenta.

Very small quantities of a protein of interest in a cell or in body fluid can be detected by an immunoassay technique called *Western blotting* (Figure 3-37). A sample is electrophoresed on an SDS polyacrylamide

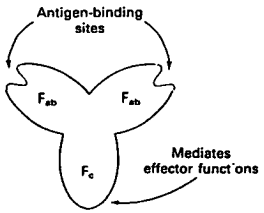


Figure 3-34
Diagram of immunoglobulin G (IgG), the major class of antibody molecules in blood plasma. IgG contains two antigen-binding F_{ab} units and an F_c unit that mediates effector functions such as the lysis of cell membranes.

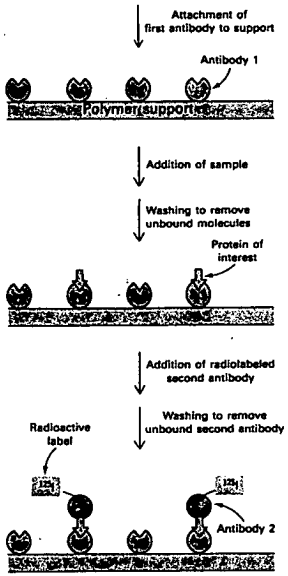


Figure 3-35
Solid-phase immunoassay. The steps are: coupling of specific antibody to a solid support, addition of the sample, washing to remove soluble compounds, and addition of a radiolabeled second antibody specific for a different site on the protein being detected.

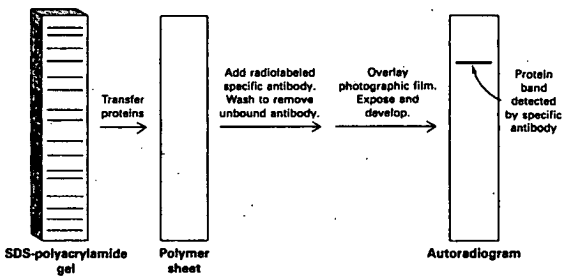


Figure 3-37
Detection of a protein on a gel by Western blotting. Proteins on an SDS-polyacrylamide gel are transferred to a polymer sheet and stained with radioactive antibody. A dark band corresponding to the protein of interest appears in the autoradiogram.

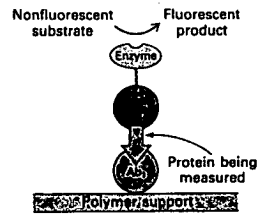


Figure 3-36
Enzyme-linked immunosorbent assay (ELISA). The steps are the same as in the immunoassay described in Figure 3-35 except that an enzyme instead of a radiolabel is attached to the second antibody. An intensely colored or fluorescent compound is formed by the catalytic action of this enzyme.

gel. The resolved proteins on the gel are transferred (by blotting) to a sheet to make them more accessible for reaction with a subsequently added antibody that is specific for the protein of interest. The antibody-antigen complex on the sheet then can be detected by rinsing the sheet with a second antibody specific for the first (e.g., goat antibody that recognizes mouse antibody). A radioactive label on the second antibody produces a dark band on x-ray film (an autoradiogram). Alternatively, an enzyme on the second antibody generates a colored product, as in the ELISA method. Western blotting makes it possible to find a protein in a complex mixture, the proverbial needle in a haystack. This technique is used advantageously in the cloning of genes (p. 133).

Antibodies are also valuable in determining the spatial distribution of antigens. Cells can be stained with fluorescent-labeled antibodies and examined by *fluorescence microscopy* to reveal the localization of a protein of interest. For example, arrays of parallel bundles are evident in cells stained with antibody specific for actin, a protein that polymerizes into filaments (Figure 3-38). Actin filaments are constituents of the cytoskeleton, the internal scaffold of cells that controls their shape and movement. The finest resolution of fluorescence microscopy is about 0.2 μm (200 nm or 2000 Å) because of the wavelength of visible light. Finer spatial resolution can be achieved by electron microscopy using antibodies tagged with electron-dense markers. For example, ferritin conjugated to an antibody can readily be visualized by electron microscopy because it contains an electron-dense core of iron hydroxide. Clusters of gold can also be conjugated to antibodies to make them highly visible under the electron microscope. *Immunoelectron microscopy* can define the position of antigens to a resolution of 10 nm (100 Å) or finer (Figure 3-39).

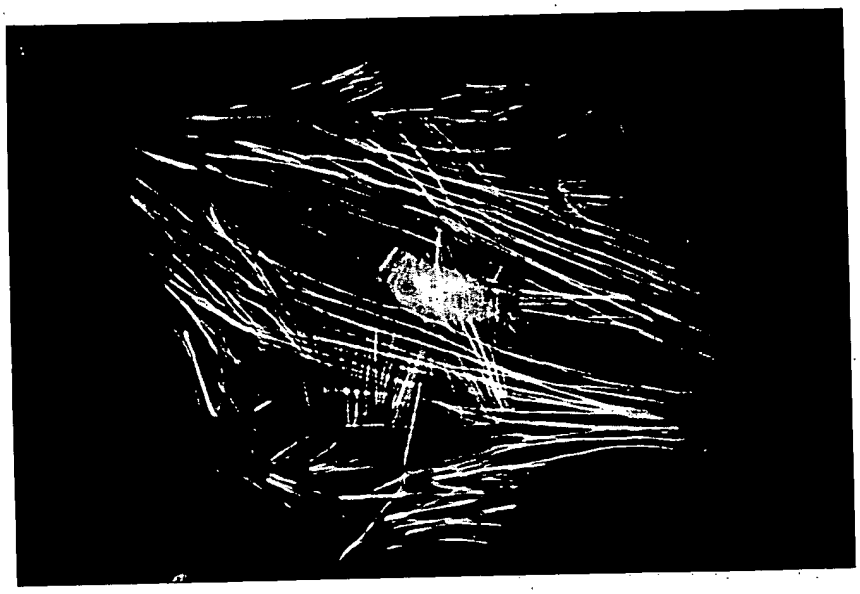


Figure 3-38
Fluorescence micrograph of actin filaments in a cell stained with an antibody specific to actin. [Courtesy of Dr. Elias Lazarides.]

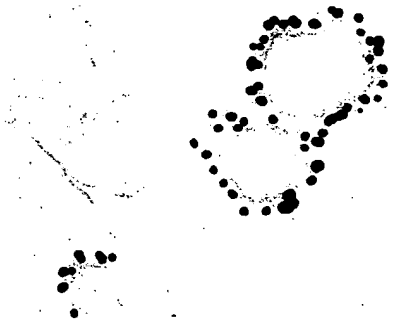


Figure 3-39
The opaque 150 Å (15 nm) diameter particles in this electron micrograph are clusters of gold atoms bound to antibody molecules. These membrane vesicles from the synapses of neurons contain a channel protein that is recognized by the specific antibody. [Courtesy of Dr. Peter Sargent.]

PEPTIDES CAN BE SYNTHESIZED BY AUTOMATED SOLID-PHASE METHODS

Synthesizing peptides of defined sequence is important for several reasons. First, *synthetic peptides can reveal the rules governing the three-dimensional conformation of proteins.* We can ask whether a particular sequence

by itself folds into an α-helix, β-strand, or hairpin turn or behaves as a random coil. Second, *many hormones and other signal molecules in nature are peptides.* For example, white blood cells are attracted to bacteria by formylmethionyl peptides that come from the breakdown of bacterial proteins. Synthetic formyl-methionyl peptides have been useful in identifying the cell-surface receptor for this class of peptides. Synthetic peptides can be attached to agarose beads to prepare affinity chromatography columns for the purification of receptor proteins that specifically recognize the peptides. Third, *synthetic peptides can serve as drugs.* Vasopressin is a peptide hormone that stimulates the reabsorption of water in the distal tubules of the kidney, leading to the formation of a more concentrated urine. Patients with diabetes insipidus are deficient in vasopressin (also called antidiuretic hormone), and so they excrete large volumes of urine (more than 5 liters per day) and are continually thirsty because of this massive loss of fluid. This defect can be treated by administering 1-desamino-8-D-arginine vasopressin, a synthetic analog of the missing hormone (Figure 3-40). This synthetic peptide is degraded in vivo much more slowly than vasopressin and, additionally, does not increase the blood pressure. Fourth, *synthetic peptides can serve as antigens to stimulate the formation of specific antibodies.*

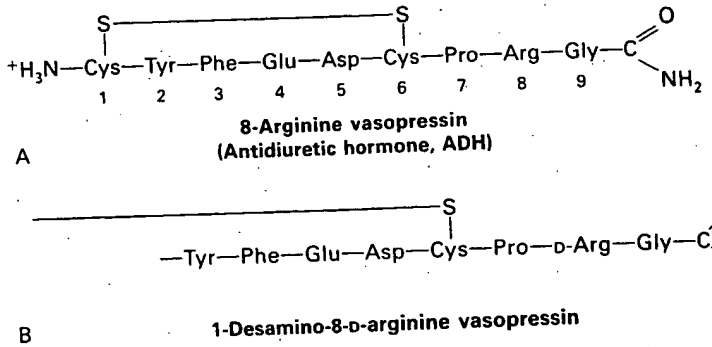
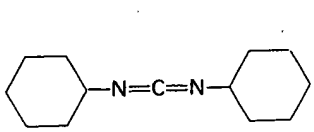
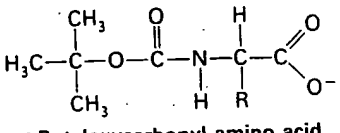
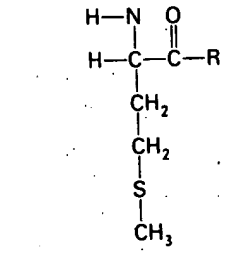


Figure 3-40
Structural formulas of (A) vasopressin (also called antidiuretic hormone), a peptide hormone that stimulates water resorption, and (B) 1-desamino-8-D-arginine vasopressin, a more stable synthetic analog.

Peptides are synthesized by linking an amino group to a carboxyl group that has been activated by reacting it with a reagent such as *dicyclohexylcarbodiimide* (DCC) (Figure 3-41). The attack of a free amino group on the activated carboxyl leads to the formation of a peptide bond and the release of dicylohexylurea. A unique product is formed only if a single amino group and a single carboxyl group are available for reaction. Hence, it is necessary to *block* (protect) all other potentially reactive groups. For example, the α-amino group of the component containing the activated carboxyl group can be blocked with a *tert*-butyloxycarbonyl (*t*-Boc) group. This *t*-Boc protecting group can be subsequently removed by exposing the peptide to dilute acid, which leaves peptide bonds intact.







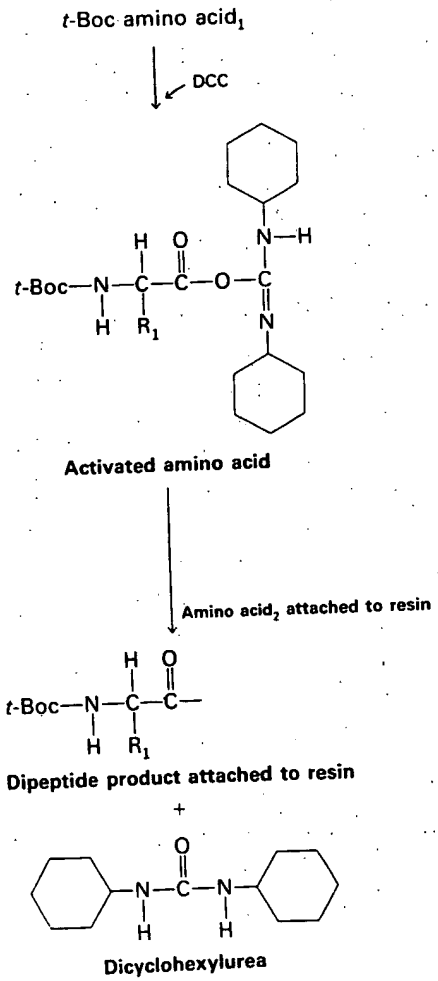


Figure 3-41
Dicyclohexylcarbodiimide is used to activate carboxyl groups for the formation of peptide bonds.

Peptides can be readily synthesized by a *solid-phase method* devised by R. Bruce Merrifield. Amino acids are added stepwise to a growing peptide chain that is linked to an insoluble matrix, such as polystyrene beads. A major advantage of this solid-phase method is that the desired product at each stage is bound to beads that can be rapidly filtered and washed and so the need to purify intermediates is obviated. All of the reactions are carried out in a single vessel, which eliminates losses due to repeated transfers of products. The carboxyl-terminal amino acid of the desired peptide sequence is first anchored to the polystyrene beads (Figure 3-42). The *t*-Boc protecting group of this amino acid is then removed. The next amino acid (in the protected *t*-Boc form) is added together with dicyclohexylcarbodiimide, the coupling agent. After formation of the peptide bond, excess reagents and dicyclohexylurea are washed away, which leaves the beads with the desired dipeptide product. Additional amino acids are linked by the same sequence of reactions. At the end of the synthesis, the peptide is released from the beads by adding HF, which cleaves the carboxyl ester anchor without disrupting peptide bonds. Protecting groups on potentially reactive side chains, such as that of lysine, are also removed at this time. This cycle of reactions can readily be automated, which makes it feasible to routinely synthesize peptides containing about 50 residues in good yield and purity. In fact, Merrifield has synthesized interferons (155 residues) that have antiviral activity and ribonuclease (124 residues) that is catalytically active.

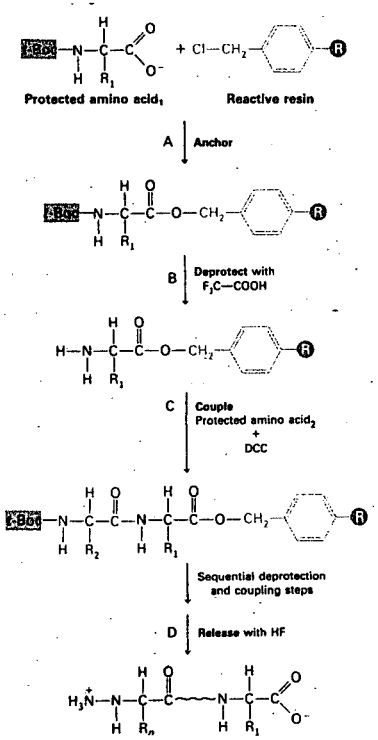


Figure 3-42
Solid-phase peptide synthesis. The steps are (A) anchoring of the C-terminal amino acid to a resin on the surface of polystyrene beads, (B) deprotection of the amino group, (C) addition of the next amino acid (in the protected form) and dicyclohexylcarbodiimide (DCC). Steps (B) and (C) are repeated for each added amino acid. The beads are washed to remove excess reagents and unwanted products after each step. Finally, (D) the completed peptide is released from the resin.

SUMMARY

The purification of a protein is an essential step in elucidating its structure and function. Proteins can be separated from each other and from other molecules on the basis of such characteristics as size, solubility, charge, and binding affinity. SDS-polyacrylamide gel electrophoresis separates the polypeptide chains of proteins under denaturing conditions largely according to mass. Proteins can also be separated electrophoretically on the basis of net charge by isoelectric focusing in a pH gradient. Ultracentrifugation and gel-filtration chromatography resolves proteins according to size, whereas ion-exchange chromatography separates them mainly on the basis of net charge. The high affinity of many proteins for specific chemical groups is exploited in affinity chromatography, in which proteins bind to columns containing beads bearing covalently linked substrates, inhibitors, or other specifically recognized groups.

The amino acid composition of a protein can be determined by hydrolyzing it into its constituent amino acids in 6 N HCl at 110°C. The amino acids can be separated by ion-exchange chromatography and quantitated by reacting them with ninhydrin or fluorescamine. Amino acid sequences can be determined by Edman degradation, which removes one amino acid at a time from the amino end of a peptide. Phenylisothiocyanate reacts with the terminal amino group to form a phenylthiocarbamoyl derivative, which cyclizes under mildly acidic conditions to give a phenylthiohydantoin (PTH)-amino acid and a peptide shorted by one residue. This PTH-amino acid is identified by high-performance liquid chromatography (HPLC). Automated repeated Edman degradations by a sequenator can analyze sequences of about fifty residues. Longer polypeptide chains are broken into shorter ones for analysis by specifically cleaving them with a reagent such as cyanogen bromide, which splits peptide bonds on the carboxyl side of methionine residues. Enzymes such as trypsin, which cleaves on the carboxyl side of lysine and arginine residues, are also very useful in splitting proteins. Recombinant DNA techniques have revolutionized amino acid sequencing. The nucleotide sequence of DNA molecules reveals the amino acid sequence of nascent proteins encoded by them but does not disclose posttranslational modifications. Amino acid sequences are rich in information concerning the kinship of proteins, their evolutionary relations, and diseases produced by mutations. Knowledge of a sequence provides valuable clues to conformation and function.

Polypeptide chains can be synthesized by automated solid-phase methods in which the carboxyl end of the growing chain is linked to an insoluble support. The α-carboxyl group of the incoming amino acid is

activated by dicyclohexylcarbodiimide and joined to the α-amino group of the growing chain. Synthetic peptides can serve as drugs and as antigens to stimulate the formation of specific antibodies. They also provide insight into relations between amino acid sequence and conformation.

Proteins can be detected and quantitated by highly specific antibodies. Enzyme-linked immunosorbent assays (ELISA) and Western blots of SDS-polyacrylamide gels are used extensively. Proteins can also be localized within cells by fluorescence microscopy and immunoelectron microscopy using labeled antibodies. X-ray crystallography has revealed the three-dimensional structures of more than a hundred proteins in atomic detail. Knowledge of molecular structure has provided insight into how proteins fold, recognize other molecules, and catalyze chemical reactions.

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PROBLEMS

1. The following reagents are often used in protein chemistry:

CNBr	Dabsyl chloride
Urea	6 N HCl
β-Mercaptoethanol	Ninhydrin
Trypsin	Phenyl isothiocyanate
Performic acid	Chymotrypsin

Which one is the best suited for accomplishing each of the following tasks?

(a) Determination of the amino acid sequence of a small peptide.
(b) Identification of the amino-terminal residue of a peptide (of which you have less than 10^{-7} g).
(c) Reversible denaturation of a protein devoid of disulfide bonds. Which additional reagent would you need if disulfide bonds were present?
(d) Hydrolysis of peptide bonds on the carboxyl side of aromatic residues.
(e) Cleavage of peptide bonds on the carboxyl side of methionines.
(f) Hydrolysis of peptide bonds on the carboxyl side of lysine and arginine residues.

2. What is the ratio of base to acid at pH 4, 5, 6, 7, and 8 for an acid with a pK of 6?

3. Anhydrous hydrazine has been used to cleave peptide bonds in proteins. What are the reaction products? How might this technique be used to identify the carboxyl-terminal amino acid?

4. The amino acid sequence of human adrenocorticotropin, a polypeptide hormone, is

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asp-Ala-Gly-Glu-Asp-Gln-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe

(a) What is the approximate net charge of this molecule at pH 7? Assume that its side chains have the pK values given in Table 2-1 (p. 21) and that the pKs of the terminal $—NH_3^+$ and —COOH groups are 7.8 and 3.6, respectively.
(b) How many peptides result from the treatment of the hormone with cyanogen bromide?

5. Ethyleneimine reacts with cysteine side chains in proteins to form *S*-aminoethyl derivatives. The peptide bonds on the carboxyl side of these modified cysteine residues are susceptible to hydrolysis by trypsin. Why?

6. The absorbance A of a solution is defined as

$$A = \log_{10} (I_0/I)$$

in which I_0 is the incident light intensity and I is the transmitted light intensity. The absorbance is related to the molar absorption coefficient (extinction coefficient) ϵ (in cm^{-1} M^{-1}), concentration c (in M), and path length l (in cm) by

$$A = \epsilon lc$$

The absorption coefficient of myoglobin at 580 nm is 15,000 cm^{-1} M^{-1}. What is the absorbance of a 1 mg/ml solution across a 1-cm path? What percentage of the incident light is transmitted by this solution?

7. Tropomyosin, a 93-kd muscle protein, sediments more slowly than does hemoglobin (65 kd). Their sedimentation coefficients are 2.6 and 4.31 S, respectively. Which structural feature of tropomyosin accounts for its slow sedimentation?

8. The relative electrophoretic mobilities of a 30-kd protein and a 92-kd protein used as standards on an SDS-polyacrylamide gel are 0.80 and 0.41, respectively. What is the apparent mass of a protein having a mobility of 0.62 on this gel?

9. The relative electrophoretic mobility of a protein on an SDS-polyacrylamide gel decreases from 0.67 to 0.64 on addition of 1 mM dithiothreitol. What is a likely reason for this shift?

10. The gene encoding a protein with a single disulfide bond undergoes a mutation that changes a serine residue into a cysteine residue. You want to find out whether the disulfide pairing in this mutant is the same as in the original protein. Propose an experiment to directly answer this question.

11. A synthetic polypeptide consisting of L-lysine residues is a random coil at pH 7 but becomes α helical as the pH is raised above 10. Account for this pH-dependent conformational transition.

12. Predict the pH dependence of the helix-coil transition of poly-L-glutamate.

Exhibit D

Flt4 Fragment Amino Acid Sequences With Stop/Start Sites Corresponding to Positions of Cyanogen Bromide Cleavage of Flt4 Receptor Tyrosine Kinase And Comprising At Least One Extracellular Domain Amino Acid

Peptide 1: 2 -Q R G A A L C L R L W L C L G L L D G L V S G Y S M - 27

Peptide 2: 28 -T P P T L N I T E E S H V I D T G D S L S I S C R G Q H P L
E W A W P G A Q E A P A T G D K D S E D T G V V R D C E G T D A R
P Y C K V L L L H E V H A N D T G S Y V C Y Y K Y I K A R I E G T T A
A S S Y V F V R D F E Q P F I N K P D T L L V N R K D A M - 154

Peptide 3: 155 -W V P C L V S I P G L N V T L R S Q S S V L W P D G Q
E V V W D D R R G M - 191

Peptide 4: 192 -L V S T P L L H D A L Y L Q C E T T W G D Q D F L S N
P F L V H I T G N E L Y D I Q L L P R K S L E L L V G E K L V L N C T
V W A E F N S G V T F D W D Y P G K Q A E R G K W V P E R R S Q Q
T H T E L S S I L T I H N V S Q H D L G S Y V C K A N N G I Q R F R E S
T E V I V H E N P F I S V E W L K G P I L E A T A G D E L V K L P V K
L A A Y P P P E F Q W Y K D G K A L S G R H S P H A L V L K E V T E
A S T G T Y T L A L W N S A A G L R R N I S L E L V V N V P P Q I H E
K E A S S P S I Y S R H S R Q A L T C T A Y G V P L P L S I Q W H W R
P W T P C K M - 468

Peptide 5: 469 -F A Q R S L R R R Q Q Q D L M - 483

Peptide 6: 484 -P Q C R D W R A V T T Q D A V N P I E S L D T W T E F
V E G K N K T V S K L V I Q N A N V S A M - 531

Peptide 7: 532 -Y K C V V S N K V G Q D E R L I Y F Y V T T I P D G F T
I E S K P S E E L L E G Q P V L L S C Q A D S Y K Y E H L R W Y R L N
L S T L H D A H G N P L L L D C K N V H L F A T P L A A S L E E V P G
A R H A T L S L S I P R V P E H E G H Y V C E V Q D R R S H D K H C H
K K Y L S V Q A L E A P R L T Q N L T D L L V N V S D S L E M - 697

Peptide 8: 698 -Q C L V G A H A P S I V W Y K D E R L L E E K S G V D
L A D S N Q K L S I Q R V R E E D A G R Y L C S V C N A K G C V N S
S A S V V E G S E D K G S M - 774

Peptide 9: 775 -E I V I L V G T G V I A V F F W V L L L L I F C N M - 800

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CONFLICT    787    787       R -> G (IN REF. 3).
CONFLICT    835    835       K -> N (IN REF. 3).
CONFLICT    848    848       V -> E (IN REF. 3).
CONFLICT   1347   1347       S -> T (IN REF. 3).
SEQUENCE   1356 AA;  151526 MW;  59E7C44B05CFEBB3 CRC64;

ry Match              38.3%;  Score 2762;  DB 1;  Length 1356;
t Local Similarity    44.1%;  Pred. No. 1.5e-175;
ches  608;  Conservative  203;  Mismatches  480;  Indels   88;  Gaps   26;

       1 MQRGAALCLRLWLCLGLLDGLVS--GYSMTPPTLNITEESHVIDTGDSLSISCRGQHPLE  58
         ||    | : ||||:      |     |:  | |:| ::   |    :| |:|||| |:
       1 MQSKVLLAVALWLCVETRAASVGLPSVSLDLPRLSIQKDILTIKANTTLQITCRGQRDLD  60

      59 WAWPGAQEAPATGDKDSEDTGVVRDC-EGTDARPYCKVLLLHEVHANDTGSYVCYYKYIK 117
         | ||  |          ||    | :| :|     :|| | : :|  ||||:| |:|:
      61 WLWPNNQSG-------SEQRVEVTECSDGL----FCKTLTIPKVIGNDTGAYKCFYR--- 106

     118 ARIEGTTAASSYVFVRDFEQPFINKPDT------LLVNRKDAMWVPCLVSIPGLNVTL-- 169
            |   |:  ||:|:|:  |||            :  |:   : :||| ||  |||:|
     107 ---ETDLASVIYVYVQDYRSPFIASVSDQHGVVYITENKNKTVVIPCLGSISNLNVSLCA 163

     170 RSQSSVLWPDGQEVVWDDRRGMLVSTPLLHDALYLQCETTWGDQDFLSNPFLVHITGNEL 229
         |       |||  : || ::|  : : ::  |  : ||    |: : |  ::| : |  :
     164 RYPEKRFVPDGNRISWDSKKGFTIPSYMISYAGMVFCEAKINDESYQSIMYIVVVVGYRI 223

     230 YDIQLLPRKSLELLVGEKLVLNCTVWAEFNSGVTFDWDYPGKQAERGKWVPERRSQQTHT 289
         ||: | |   :|| ||||||||||   | | |: |:|:|| : :  | |      |: :
     224 YDVVLSPSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGS 283

     290 ELS---SILTIHNVSQHDLGSYVCKANNGIQRFRESTEVIVHENPFISVEWLKGPILEAT 346
         |:    | |||  |:: | | | | |::|:   : || | ||| ||::       ::|||
     284 EMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEKPFVAFGSGMESLVEAT 343

     347 AGDELVKLPVKLAAYPPPEFQWYKDGKALSGRH---SPHALVLKEVTEASTGTYTLALWN 403
         | | |::| |   ||||| :|||:|  |   |   : | | : ||:|  || ||: | |
     344 VG-ERVRIPAKYLGYPPPEIKWYKNGIPLESNHTIKAGHVLTIMEVSERDTGNYTVILTN 402

     404 SAAGLRRNISLELVVNVPPQIHEKEASSP-SIYSRHSRQALTCTAYGVPLPLSIQWHWRP 462
           :  :::  : ||| ||||| ||   ||   |   : | |||| | :| |  | |:|:
     403 PISKEKQSHVVSLVVYVPPQIGEKSLISPVDSYQYGTTQTLTCTVYAIPPPHHIHWYWQL 462

     463 WTPCKMFAQRSLRRRQQQDLMPQCRDWRAVTTQDAVNPIESLDTWTEFVEGKNKTVSKLV 522
         |   |    :     :  | | :||:|      | ||        :|||||||| ||
     463 EEEC---ANEPSQAVSVTNPYP-CEEWRSVEDFQGGNKIEVNKNQFALIEGKNKTVSTLV 518

     523 IQNANVSAMYKCVVSNKVGQDERLIYFYVTTIPDGFTIESKPSEELLEGQPVLLSCQADS 582
         || |||||:|||   ||||: ||:| |:||  |:   |  :|  :  | : | | | ||
     519 IQAANVSALYKCEAVNKVGRGERVISFHVTRGPE---ITLQPDMQPTEQESVSLWCTADR 575

     583 YKYEHLRWYRLNLSTLHDAHGNPLLLDCKNV----HLFATPLAASLEEVAPGARHATLSL 638
           :|:| ||:|    |    |      |||:     | ||  : |  ::        | :
     576 STFENLTWYKLGPQPLPIHVGELPTPVCKNLDTLWKLNATMFSNSTNDI--------LIM 627

     639 SIPRVAPEHEGHYVCEVQDRRSHDKHCHKKYLSVQALEAPRLTQNLTDLLVNVSDSLEMQ 698
         :   : : :| |||  |||::  :||  : |:|    || :| || :   :: :|:|:
     628 ELKNASLQDQGDYVCLAQDRKTKKRHCVVRQLTVLERVAPTITGNLENQTTSIGESIEVS 687

     699 CLVAGAHAPSIVWYKDERLLEEKSGVDLADSNQKLSIQRVREEDAGRYLCSVCNAKGCVN 758
         |  :|   | |:|:||   | | ||: | | |: |:|:|||:|| | | |  |:  ||
     688 CTASGNPPPQIMWFKDNETLVEDSGIVLKDGNRNLTIRRVRKEDEGLYTCQACSVLGCAK 747

     759 SSASVAVEGSEDKGSMEIVILVGTGVIAVFFWVLLLLIFCNMRRPAHADIKTGYLSIIMD 818
           |   :||:::| ::||:|||| |||:|||:||::|   ::|    ::|||||||:||
     748 VEAPPIIEGAQEKTNLEIIILVGTAVIAMFFWILLVIILRTVKRANGGELKTGYLSIVMD 807
```

EXHIBIT E